QUANTIFYING RISK FOR THE NORTHERN ROOT-KNOT NEMATODE, *MELOIDOGYNE HAPLA*, IN POTATO IN NEW YORK STATE

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The Northern root-knot nematode, *Meloidogyne hapla*, is an important soilborne pathogen of potato in New York State. Initial pathogen populations (P_i) are often inversely correlated with crop loss and damage, with the economic threshold representing the P_i likely to result in significant crop loss, thus justifying use of control tactics. However, the economic threshold for *M. hapla* in potato in New York State is not well understood. Furthermore, standard methods of enumerating populations of nematodes from soil are time consuming and error prone. This research sought to quantify the risk of crop loss associated with *M. hapla* P_i, and develop a DNA-based soil test for prediction of risk. A method for isolating nematode DNA from 100 g of soil using super paramagnetic iron oxide nanoparticles was developed and tested using artificially inoculated soil and field collected samples. A species-specific, real-time quantitative PCR (qPCR) assay targeting the root-knot nematode effector gene *16D10* was developed for quantifying *M. hapla*. Spatial and spatiotemporal analysis of *M. hapla* and root-lesion nematodes, *Pratylenchus* spp., was conducted by intensively sampling three commercial potato fields in New York State. This informed enhanced sampling strategies and evaluated the potential for site-specific nematicide application. The influence of *M. hapla* P_i on crop loss was assessed by quantifying *M. hapla* P_i in commercial potato fields through manual extraction and a nested qPCR assay, and

investigating associations with yield components. *M. hapla* P_i did not have a significant effect on total yield, number of tubers, tuber diameter, or tuber galling severity, suggesting the economic threshold for *M. hapla* in potato is higher than previously regarded (approximately 100 *M. hapla* second-stage juveniles (J2s) per 100 g soil), and/or tolerant cultivars were used. The response of the commercial potato cvs. Eva and Lamoka to differing P_i of *M. hapla* and *P. penetrans* was assessed through replicated field trials. Yield was not impacted even at the highest P_i tested (500 *M. hapla* J2s or 1,000 *P. penetrans* nematodes). These results will be used to guide future research to refine economic thresholds, explore advanced nematode management options, and assess potato cultivar susceptibility to plant-parasitic nematodes.

BIOGRAPHICAL SKETCH

Adrienne Marie Gorny was born in Cleveland, Ohio on June 25, 1992. She grew up in Canton, Michigan, where she attend the fledging All Saints Catholic School, and later attended Ladywood High School in Livonia, Michigan. From her Advanced Placement Biology class at Ladywood, she was drawn to studying plant science, and in 2010, began her undergraduate studies at Purdue University in West Lafayette, Indiana, where she majored in Plant Biology. From her mentors and classes at Purdue, she developed a focused interest in plant pathology, and participated in the Cornell University Summer Scholars program at the New York State Agricultural Experiment Station in 2013, which reaffirmed this interest. After graduating with college honors from Purdue in 2014, she returned to Cornell University to begin as an M.S./Ph.D. student in the laboratory of Dr. Sarah Pethybridge at Cornell AgriTech in Geneva, New York State, within the Section of Plant Pathology and Plant-Microbe Biology. At Cornell, her research centered on the quantitative epidemiology of the Northern root-knot nematode, *Meloidogyne hapla*, in potato.

To my parents

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CHAPTER 1

INTRODUCTION

Potato production in New York State and plant-parasitic nematodes

Potatoes are an economically important vegetable crop in New York State, with the majority of production occurring in the Western and Central regions, and Long Island, where rich glacial mineral soils and high organic matter 'muck' soils are found (L. Stivers, Cornell CE). A broad range of white, red, and yellow cultivars are grown primarily for the fresh (tablestock) and processing (chipping) markets, including specialty types such as fingerling and blue skinned potatoes (L. Stivers, Cornell CE). New York State also produces seed potatoes, with Cornell University's College of Agriculture and Life Sciences serving as the seed certification agency for the state (Anonymous 2017). In 2018, New York State farms harvested approximately 5,900 hectares of potatoes worth approximately $49.8 million USD (USDA-NASS 2019).

Potato is host to many species of plant-parasitic nematodes, which are microscopic, soilborne, non-segmented roundworms belonging to the Kingdom Animalia, phylum Nematoda. Plant-parasitic nematodes may affect potato production by limiting plant growth and reducing marketable tuber yields. The most destructive nematodes affecting potato production include potato cyst (*Globodera* spp.), root-knot (*Meloidogyne* spp.), root-lesion (*Pratylenchus* spp.), and potato rot nematode (*Ditylenchus destructor*) (Mai et al. 1981; Bridge and Starr 2007; Fiers et al. 2012). Nematode feeding damage on the roots may also provide entry points for bacterial and fungal pathogens, leading to secondary infections and tuber rots (Fiers et al. 2012).

Potato cyst nematodes (*Globodera* spp.) are sedentary endoparasites, feeding at a fixed site known as a syncytium (Bridge and Starr 2007). Juvenile stages molt inside the host

root tissues, after which enlarged females will break through the root epidermis and form a hardened and desiccation-resistant cyst (Mai et al. 1981; Bridge and Starr 2007). Cysts may be observed on infected roots and in surrounding soil. Two important species of potato cyst nematode are *G. rostochiensis* (golden cyst) and *G. pallida* (pale cyst). The species may be visually distinguished from each other by the color of the females' bodies after they have emerged from the root tissue, but before they reach maturity. The female bodies of *G. rostochiensis* and *G. pallida* are yellow to gold and milky-white, respectively, prior to maturity at which time they are both brown (Mai et al. 1981). Both *G. rostochiensis* and *G. pallida* have been found in New York State and are quarantined pathogens, primarily isolated to Cayuga, Nassau, Seneca, Steuben, Suffolk, and Wayne counties (USDA-APHIS 2019). Symptoms of disease caused by potato cyst nematodes include stunting, general decline, or symptoms resembling water stress (Mai et al. 1981).

Root-lesion nematodes (*Pratylenchus* spp.) are important soilborne pathogens of many vegetable crops (Olthof and Potter 1973; Potter and Olthof 1974), with *P. penetrans* being the most damaging species within the genus to potato (Mai et al. 1981; Kotcon et al. 1987; France and Brodie 1995). *P. penetrans* is a migratory endoparasite, with second-stage juveniles (J2s) through vermiform (motile, worm-like) adults capable of penetrating host roots immediately behind the root cap, and migrating through root tissues to feed (Bridge and Starr 2007). Root-lesion nematode feeding results in necrotic lesions and root dieback. *P. penetrans* is primarily a pathogen of the roots (Mai et al. 1981), however, on tubers it may produce necrotic surface lesions that are similar in appearance to common scab caused by the bacterial pathogens *Streptomyces* spp. (Holgado et al. 2009). *P. penetrans* has also been implicated in the potato early dying disease complex with the fungal pathogen *Verticillium dahliae* (MacGuidwin and Rouse 1990).

The potato rot nematode (*Ditylenchus destructor*) is a migratory endoparasite of potatoes and other plants, particularly those with tubers, rhizomes, bulbs, or other enlarged below ground structures, including carrot, sugar beet, and iris (Sturhan and Brzeski 1991). In potato, *D. destructor* enters the tuber through enlarged lenticels or other wounds, and symptoms include small white lesions in the tuber flesh, which may become necrotic as secondary pathogens invade. Generally no specific aboveground symptoms are observed (Mai et al. 1981). Control of potato rot nematode is best achieved by using certified clean seed to avoid introduction. Dry heat treatment of seed potatoes has been used with some success to disinfect planting materials (Sturhan and Brzeski 1991). *D. destructor* is not known to be present in New York State (CABI Invasive Species Compendium 2018).

Root-knot nematodes

Root-knot nematodes (RKN; *Meloidogyne* spp.) are obligate soilborne plant pathogens in the order Tylenchida, phylum Nematoda (Perry et al. 2009; Maggenti 1991). They are sedentary endoparasites which begin their lifecycle as eggs (Fig. 1.1A), molt to first stage juveniles within the egg (Fig. 1.1B), and hatch as an infectious second-stage juveniles (J2s; Fig. 1.1C). The J2s penetrate host roots immediately behind the root cap, and use a suite of effectors secreted from the stylet (Jones et al. 2013) (Fig. 1.1D), to establish specialized feeding sites composed of giant cells. The J2s then passes through two further juvenile stages (Fig. 1.1E) to become adults. Adult females swell in size to become pear-shaped (Fig. 1.1F), releasing eggs in a gelatinous matrix outside the root surface (Perry et al. 2009; Jones et al. 2013) (Fig. 1.1G). Temperature and moisture promote egg hatching and the emerging J2s may re-infect the same host plant (Perry et al. 2009). Males and J2s therefore constitute the motile, vermiform stages of the lifecycle.

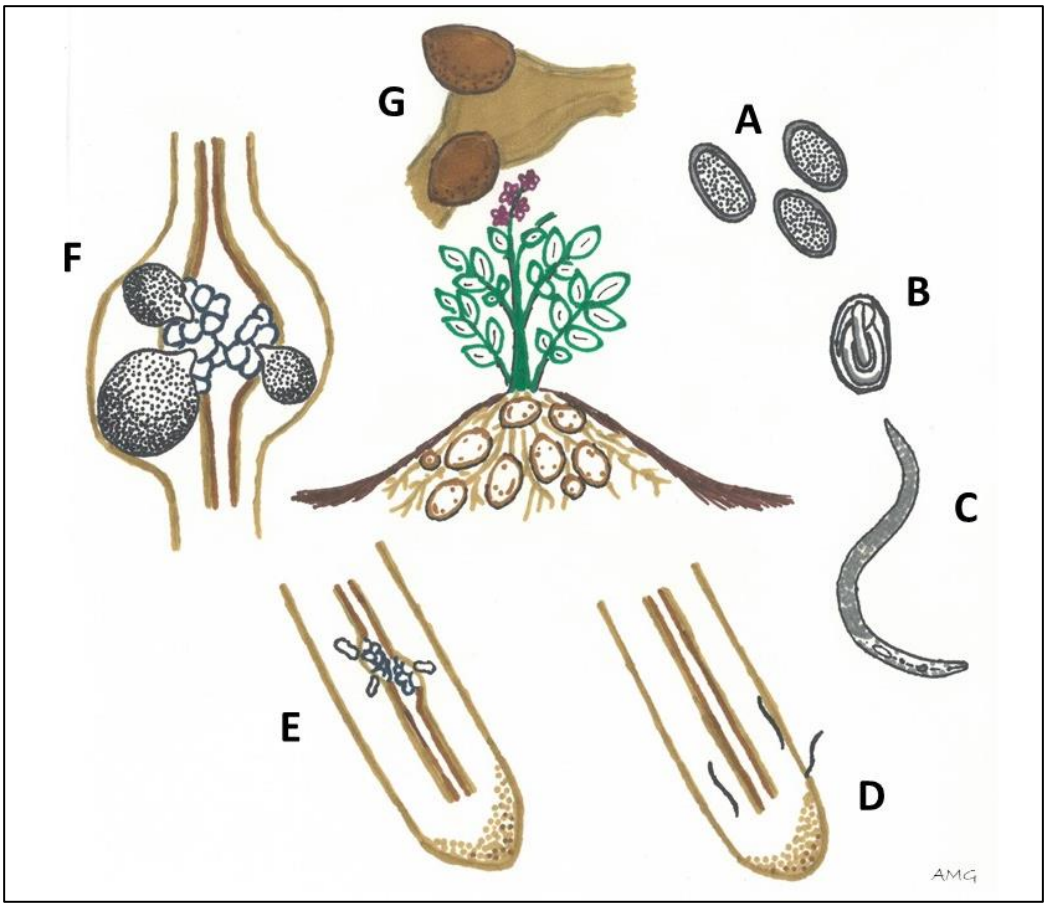

Fig. 1.1. Lifecycle of the Northern root-knot nematode, *Meloidogyne hapla* on potato. The lifecycle begins as an egg (**A**), which is laid outside of the root. Root-knot nematodes molt to the first-stage juvenile within the egg (**B**), then hatch as an infectious second-stage juvenile (**C**). Second-stage juveniles locate and penetrate host roots in the zone of elongation (**D**), just behind the root cap, and establish a fixed feeding site composed of giant cells formed by the action of secreted effectors (**E**). Adult females increase in size (**F**), and eggs are extruded in a gelatinous egg sack outside the root, which turn brown as they mature (**G**). *Illustration: A. Gorny.*

Signs of RKN infection include motile J2s (Fig. 1.2A) and males that may be visualized under magnification following extraction from soil or plant tissues, and adult females embedded within roots (Fig. 1.2B). Symptoms of RKN infection in potato may manifest as galls or knots on fine root hairs (Fig. 1.2C) with brown-colored egg sacks protruding from the surface of the galls, resulting in reduced root function and

nondescript aboveground symptoms such as chlorosis, wilting, stunting, and uneven crop stands (Taylor and Sasser 1978; Duncan and Phillips 2009). Infection may also occur in the tubers, with females usually found only a few millimeters below the surface (Mitkowski and Abawi 2003) (Fig. 1.2D).

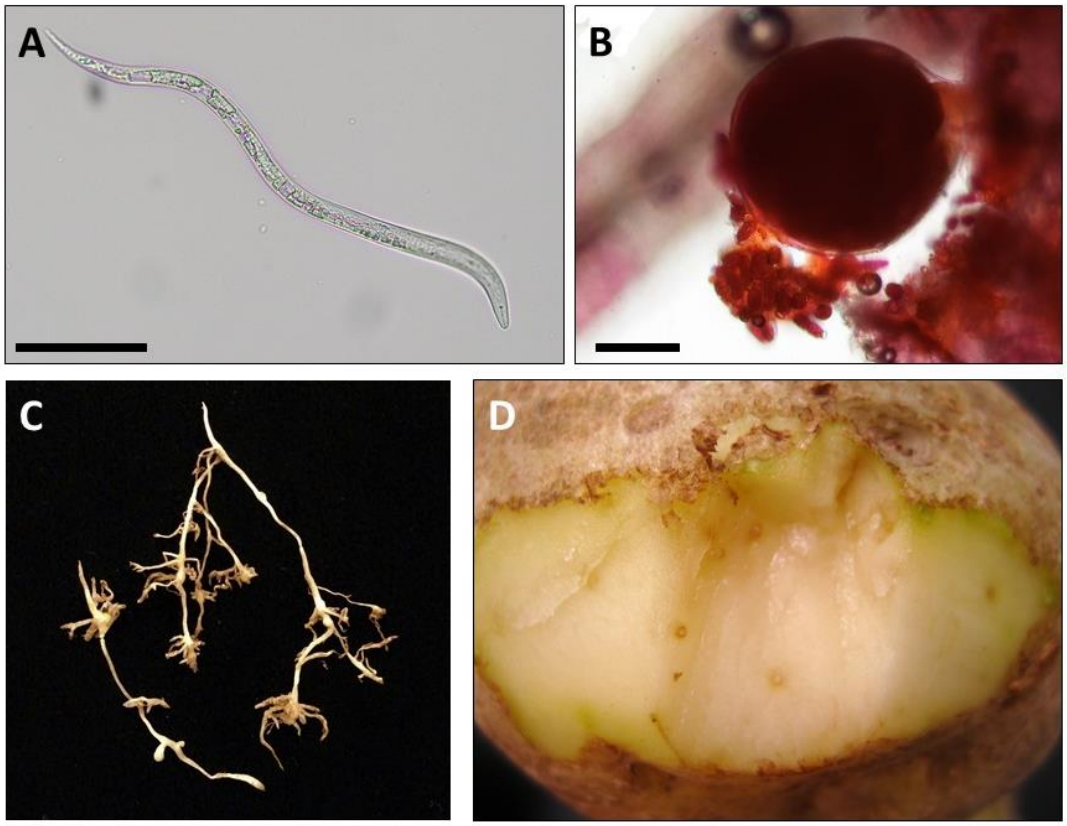

Fig. 1.2. Signs and symptoms of the Northern root-knot nematode (*Meloidogyne hapla*) on potato include motile second-stage juveniles (**A**, scale = 50 µm), enlarged adult females with eggs extruding from roots (**B**, scale = 200 µm), knotting and galling on roots (**C**), and adult females embedded within a tuber (**D**).

RKN are associated with substantial reductions in yield and quality in potato worldwide (Brodie et al. 1993). Reductions in yield vary with crop, production region, species, and population density in the soil. Several RKN species affect potato, with the most globally significant including the peanut RKN (*M. arenaria*), Columbia RKN

(*M. chitwoodi*), false Columbia RKN (*M. fallax*), Northern RKN (*M. hapla*), Southern RKN (*M. incognita*), and Javanese RKN (*M. javanica*) (Brodie et al. 1993; Volvas et al. 2005). In the western United States, *M. chitwoodi* may cause extensive physical defects to potato tubers, rendering the crop unacceptable for sale in the fresh and processing markets (Ingham et al. 2007). In Idaho, fresh market potato tubers may be rejected if as little as 5% of tubers have damage from *M. chitwoodi* (King and Taberna 2013).

In potato, RKN infestation may cause yield loss through compromising root function and limiting plant growth, resulting in reduced tuber size or set, or through direct physical damage to tubers, rendering them unmarketable due to reduced aesthetics from blemishes on the tuber surface (Gudmestad et al. 2007; Fiers et al. 2012). Yet, economic losses from RKN damage are not limited to simple yield reductions. Additional costs of sampling and diagnostics, chemical treatments and sanitation measures, increased need of irrigation and fertilizers due to RKN galling resulting in compromised root function and suboptimal plant growth, restricted crop rotation choices, and quarantine regulations also reduce farm profit and sustainability (Jones et al. 2013; Wesemael et al. 2014).

The Northern RKN, *Meloidogyne hapla* Chitwood, 1949, has worldwide distribution, yet is particularly problematic in temperate climates (Perry et al. 2009), including the Northeastern United States. In New York State, *M. hapla* is considered the only RKN species in agricultural fields (Mitkowski et al. 2002). *M. hapla* has a broad host range and causes significant reductions in yield and quality on many economically important annual crops including *Solanum* spp. (Van der Beek et al. 1998), carrot (Vrain 1982; Sapkota et al. 2016), table beet (Potter and Olthof 1974), lettuce (Olthof and Potter 1972), tomato (Olthof and Potter 1977), and soybean (Barker and Olthof 1976), and perennial crops, including grapevine (Walker 1997) and coffee (Handoo et al. 2005). In spinach, Potter and Olthof (1974) measured a yield reduction of nearly 30% in microplots infested with an initial density of 2,000 *M. hapla* nematodes per kg of soil.

Quantification of crop damage and prediction of risk

Pedigo et al. (1986) defines crop *injury* as the harmful action of a pest or pathogen on a host crop, and *damage* as the subsequent loss of host utility, including yield loss and reductions in quality. Knowledge of RKN population densities in the soil and the corresponding damage resultant from those populations enables the calculation of economic injury levels. The *economic injury level* is the lowest or minimum pest population density that will cause *economic loss*, defined as the crop damage that justifies the cost of pest control measures (Stern et al. 1959). Southwood and Norton (1973) further offer the similar term *economic damage* as the point at which crop damage from pest injury equals the cost of implementing control measures. This definition is tightly linked to the *economic threshold*, defined by Stern et al. (1959) as the pest or pathogen population density at which control measures should be implemented to hinder the population from attaining the economic injury level. Stern et al. (1959) notes the economic threshold is set lower than the economic injury level to permit a reasonable timeframe for implementing control measures. Understanding of crop loss at the field level, and the economic threshold for a pathogen within the crop can inform short-term control tactics (such as chemical application or selection of tolerant cultivars) and long-term management strategies (such as guiding research priorities and breeding programs) (Savary et al. 2006).

Economic thresholds are a key component of *decision theory*, which describes the use of mathematical paradigms to underpin optimal decision-making when potential options involve risk of gain or loss. The decisions made in a crop protection scenario are a function of the growers' goals, their perception of risk, the resource limitation of tactics available for use, and the decision rules used for action (Norton 1976). Decision theory in plant disease management is operationalized by disease predictive systems (Gent et

al. 2013). These systems are driven by risk algorithms, which are mathematical tools that appraise disease risk factors to inform users when action (evasive or protective measures) may be required (Krause and Massie 1975; Hughes et al. 1999; Gent et al. 2013). In many pathosystems, the presence of inoculum is assumed and factors including weather conditions, host plant susceptibility, and disease history are quantified in a forecasting index to determine whether an infection period has occurred and if pesticide use is warranted (Hughes et al. 1999). The holistic goal of these systems is to determine when pesticides are truly needed, thus avoiding unnecessary applications.

Predicting risk for soilborne diseases, such as those caused by plant-parasitic nematodes, is strongly reliant upon effectively quantifying the presence of inoculum within a field (Madden et al. 2007) and quantification of populations in soil prior to planting has led to an enhanced understanding of how inoculum density influences crop loss (Ophel-Keller et al. 2008; Bilodeau 2011). The quantitative relationship between nematodes and yield of an annual crop is a function of pre-plant population densities (Seinhorst 1965). For RKN in many cropping systems, population densities prior to planting and crop damage are significantly inversely correlated (Seinhorst 1965; Barker and Olthof 1976). Moreover high populations of RKN in the soil prior to, or at, planting is likely to lead to an increased infection rate during initial stages of the epidemic, resulting in elevated disease severity (crop damage) throughout the growing season (Madden et al. 2007). The relationship between population density and yield loss has been described for *M. hapla* in many pathosystems including potato (Wheeler et al. 1994), tomato (Olthof and Potter 1972; Barker et al. 1976), and carrot (Gugino et al. 2006). It follows then that accurate pathogen detection, quantification, and understanding of the association between population density and crop loss is important for supporting informed disease management decisions and crop loss prediction models for *M. hapla* and other plant-parasitic nematodes.

Root-knot nematode management

Management of nematodes within a crop production system may be operationalized into *strategies* and *tactics*. Disease management strategies are the goals and objectives desired as the outcome of the management plan, while disease management tactics are the actions and operations used to implement the strategy and bring about a positive outcome (Arneson 2001). For example, one strategy may be to reduce plant-parasitic nematode population densities to below the economic injury levels, while another strategy may be to avoid introduction to the farm. Tactics to achieve these strategies may include planting a biofumigant crop or application of pesticides to reduce populations, and planting certified disease-free material, or washing farm equipment to reduce movement of infested soil.

Decisions regarding plant disease management in intensively managed agricultural systems are integrated functions of crop loss risk and, for RKN, are driven by assumptions of the ubiquitous presence of inoculum (i.e. nematode individuals and eggs), making the strategy of reducing inoculum within the field of primary importance. The goal of this strategy is to reduce initial RKN populations to below the pre-plant economic threshold to limit the damage in the following crop (Barker and Olthof 1976; Madden et al. 2007). Tactics used within this strategy may be implemented either within season or prior to planting the crop. Some researchers have noted that although highly effective single control tactics have been used, an integrated approach that utilizes two or more strategies should ultimately be considered for durable control (Roberts 1993).

Within season tactics for reducing inoculum may include crop rotation, resistant varieties and cultivars, or the application of systemic nematicides. Crop rotation is the alternation of host and non-host crops in a spatial or temporal sequence to reduce nematode populations by withholding a suitable host for a certain length of time to

inhibit feeding and reproduction (Trivedi and Barker 1986). Knowledge of the species of nematodes present (even including race or pathotype) and the host status of the rotation crops is essential for building a robust and successful rotational program (Trivedi and Barker 1986). Although RKN species are generally polyphagous and have a broad host range thus making selection of rotational crops difficult (Duncan 1991), certain grain crops (e.g., maize and oats) and grasses are poor hosts of RKN (Trivedi and Barker 1986; Duncan 1991; Bridge 1996).

The planting of varieties and cultivars with genetic resistance to nematodes is another tactic with utility for control of sedentary nematodes by inducing the hypersensitive response (Duncan 1991; Castagnone-Sereno 2002). For example, the tomato resistance gene *Mi* confers strong resistance to the RKN species *M. arenaria*, *M. incognita*, and *M. javanica* (Gururani et al. 2012). However, with the exception of tomato, few highly sensitive crops have significant practical resistance to RKN species (Roberts 1992) and no known genetic resistance to *M. hapla* is available in commercial cultivars of potato (Janssen et al. 1995; Van der Beek et al. 1998; Melakeberhan et al. 2007). The absence of commercially acceptable cultivars with enhanced genetic resistance to RKN and practical challenges in effective management through crop rotation means that populations are frequently controlled by applications of systemic, non-fumigant nematicides (e.g., oxamyl and fluopyram), either at planting and/or during the cropping season.

Other management tactics must be undertaken prior to planting of the main crop, as they are either phytotoxic or require the soil to be free of host plants. These tactics include soil fumigation, biofumigation, and field fallow. Soil fumigation is the application of a fumigant nematicide (e.g., methyl bromide, metam sodium, and 1,3-dichloropropane) for suppression of plant-parasitic nematodes and other soilborne pests. Fumigant nematicides are commonly applied as liquids, which volatize and disperse into

air and water pockets within the soil, delivering toxic levels of the active ingredients to the nematodes as the compound moves upwards through the soil profile (Lembright 1990). Several factors affect the success of nematode control through fumigation, including soil type, moisture content, temperature, percent organic matter, and diffusion coefficient of the product (Lembright 1990; Chitwood 2003). Overall, toxic levels of the product must contact the nematode for an adequate length of time in order for sufficient control to occur (Noling 1997).

Biofumigation is a technique in which plant-parasitic nematodes are suppressed by the planting and incorporation of a cover crop containing nematicidal compounds into the soil as a green manure. As the living tissues break down in the soil, the nematicidal compounds are liberated and dispersed through the soil (Ploeg 2008; Kruger et al. 2013). Examples of biofumigant crops include certain *Brassica* spp. that produce glucosinolate compounds, which hydrolyze to nematode-toxic isothiocyanates and thiocyanates as the tissues break down (Ploeg 2008). The selection of an appropriate biofumigant crop and timing of incorporation are critical for the success of this method, and elevated levels of glucosinolates within the crop tissues may not necessarily correlate with increased levels of nematode control (Ploeg 2008).

Another pre-season control tactic is field fallow, a technique in which the field is taken out of production and removed of vegetation through tillage or herbicides for a certain length of time (Trivedi and Barker 1986). Field fallow accomplishes population decline by withholding suitable host plants, causing death of the plant-parasitic nematodes by starvation and reducing reproductive success (Trivedi and Barker 1986). RKN populations may be controlled through a grass fallow, in which the field is devoid of broadleaf plants and planted to solely grasses (which are poor or non-hosts of RKN) (Bridge 1996). Control of weeds within the fallowed field is of high importance, as many species are alternative hosts of RKN (Rich et al. 2008).

Nematode enumeration

Due to the association between nematode population densities and crop yield, and the cost of implementing certain control tactics, knowledge of population densities within the soil is imperative for supporting informed management decisions. Nematode populations may be adequately predicted through standard nematode enumeration techniques (Olthof and Potter 1972; Wheeler et al. 1994), including the Baermann funnel and various modifications (Baermann 1917; Overgaard-Nielsen 1949; Christie and Perry 1951), Whitehead trays (Whitehead and Hemming 1965), and elutriation (Seinhorst 1956). These techniques involve extracting vermiform, cysts, or eggs from a determined volume of soil using specialized apparatus, followed by quantification through identification at high power magnification (e.g., 100 to 400×). These standard techniques typically only extract motile nematodes, thus representing only a portion of potential infectious units. Identification of extracted nematodes is performed by evaluating morphological features, and can be a technically difficult process, requiring identification by an experienced nematologist (Min et al. 2012; Sapkota et al. 2016). Even with this, many specimens can only be accurately identified to genus (Sawada et al. 2011) making detailed management recommendations challenging.

Another standard technique used for quantifying nematode population density and disease risk is the bioassay, a method where field soil is placed in pots and sensitive indicator plants (e.g., carrot and tomato for RKN) are grown. High disease severity in the indicator plants is associated with high population densities and the results inform management decisions (Gugino et al. 2006). However, greenhouse bioassays are technically difficult and time-consuming, as indicator plants must be grown for a sufficient period of time (usually weeks to months) for disease or damage to develop. Bioassays are frequently limited to assessment of a single pathogen type, and species

and race level information is dependent on the type of indicator plant selected. Thus, bioassays do not facilitate time-sensitive, pathogen-specific management decisions.

Due to the aforementioned difficulties of standard enumeration techniques, nematode management decisions are usually made in the absence of data on inoculum densities, and thus decisions are often risk averse and prophylactic in nature, calling for the application of costly preventative nematicide treatments before or at planting. This tactic may result in false positive decisions, where a nematicide is applied but RKN populations were below the economic threshold. This approach increases variable costs of production, maintains selection pressure on pathogen populations in the environment, increases the probability of resistance development or amplifies biodegradation of the compound by soil microorganisms, and potentially has off-site deleterious effects on the environment.

Prediction of damage from RKN may therefore be substantially improved by reforming diagnostic soil tests, the results of which are provided prior to planting and are highly sensitive and specific for pathogen species relevant to the planned crop. Results of these pre-plant soil tests may inform disease management strategies, such as crop rotations or selection of resistance or tolerant crop varieties, or management tactics, such as pesticide and fumigant applications. Rapid depiction of the spatial distribution and heterogeneity in nematode population densities across a field may also enable variable application of pesticides, promoting conservative and efficient use.

Knowledge of pre-plant, plant-parasitic nematode population densities, although a key component of the risk estimate equation, is not solely sufficient for highly accurate economic thresholds (Melakeberhan et al. 2012). Other abiotic factors, including soil type and environmental conditions, are also likely important to crop loss risk evaluation. For example, Ferris (1985) showed that maximum RKN reproductive factors occurred in coarse soil, compared to those in soil with higher amounts of fine clay particles.

Galling and deformation from *M. hapla* has also been reported to be more severe in potato when soil temperatures were high (20°C) (Griffin and Jorgenson 1969; Stirling and Wachtel 1985). Although inclusion of additional edaphic variables can potentially lead to a more robust prediction model, these abiotic factors were not systematically investigated within this thesis.

DNA-based detection and quantification assays

Recent advances in molecular biology offer a transformational step-change for risk prediction of soilborne diseases by quantifying DNA as a proxy for population densities. Quantitative polymerase chain reaction (qPCR) is one method that has gained popularity due to the ability to both detect and quantify pathogens of interest. This method involves the design of primers that are specific to a target region of interest and the incorporation of a fluorogenic intercalating dye. As amplification proceeds, increases in dye activity due to increased target generation is quantified within a real-time quantitative PCR thermocycler platform. The quantity of fluorescence may be compared to a standard curve of known samples to calculate quantities in unknown samples (Arya et al. 2005). Numerous qPCR protocols have been developed to detect and quantify populations of important plant-parasitic nematode species important in potato production, including *Globodera pallida* (Mandani et al. 2005), *G. rostochiensis* (Toyota et al. 2008; Goto et al. 2010), *P. thornei* (Yan et al. 2012), *M. fallax* (Hay et al. 2016), *M. enterolobii* (Braun-Kiewnick et al. 2016), and *Ditylenchus* spp. (Jeszke et al. 2015). These qPCR protocols have proven significantly faster and more precise in distinguishing species than traditional enumeration techniques (Yan et al. 2012; Ahmed et al. 2016; Braun-Kiewnick and Kiewnick 2018) and many samples may be run in parallel, making the method rapid and cost effective.

Critical to the success of qPCR is a highly specific amplification target. Several published qPCR methods utilize the internal transcribed spacer (ITS) ribosomal DNA (rDNA) region as a target for amplification (e.g., *G rostochiensis* (Toyota et al. 2008; Goto et al. 2010), *M. chitwoodi* (Zijlstra and Van Hoof 2006), and *P. neglectus* (Yan et al. 2013)). However, intraspecies variability and a high degree of heterogeneity of the ITS rDNA region has been observed in many plant-parasitic nematode species, including *Meloidogyne* spp. (Zijlstra et al. 1995; Powers et al. 1997; Hafez et al. 1999; Hugall et al. 1999; Anthoine and Mugniéry 2005; Huang et al. 2010; Prospero et al. 2015; Jeszke et al. 2015).

Effector genes encode small proteins essential to parasitism (Smant and Jones 2011; Haegeman et al. 2012; Mitchum et al. 2013; Rehman et al. 2016). Many effectors are specific to species (Smant and Jones 2011; Haegeman et al. 2012) and could be harnessed as new targets for qPCR amplification, creating highly species-specific assays for molecular diagnostics. For example, a specific and sensitive assay for detection of the root-lesion nematodes *P. penetrans* and *P. thornei* was designed using the β-1,4-endoglucanase gene (Mokrini et al. 2013; 2014). This effector gene encodes for a secreted cellulase used for aiding host root penetration and intracellular migration (Wang et al. 1999; Gao et al. 2004).

Isolation of nematode DNA from soil

Prior to detection and quantification of nematode populations by molecular analysis, a robust and reliable method for isolating DNA from soil is required. Isolating high quality DNA directly from microorganisms in soil is difficult due to the presence of contaminants such as humic and phenolic compounds, polysaccharides, and proteins that are frequently co-extracted with DNA. These contaminants may inhibit polymerase activity and interfere with efficient amplification and fluorescence signals from qPCR

or other molecular assays. Furthermore, isolating DNA from nematode bodies may be challenging due to the cuticle being highly recalcitrant to common lysis materials (Bird and Bird 1991; Johnstone 1994). Numerous protocols for DNA extraction from soil have been described (e.g., Steffan et al. 1988; Purdy et al. 1996; Yan et al. 2008; Kathiravan et al. 2015), and although these protocols produced high quality nematode DNA, they frequently require phenol or chloroform, rendering them hazardous to the operator and environment (Bandyopadhyay et al. 2011). Commercial DNA extraction kits do not produce hazardous chemical waste. However, many kits are cost prohibitive and limited to processing only small volumes of soil (often only 0.25 to 10 g of soil) which introduces uncertainty (due to spatial heterogeneity of the pathogen) when attempting to translate results to field level management recommendations (McSorley and Walter 1991; Hay et al. 2016). A DNA isolation method capable of extracting nematode DNA from large volumes (e.g., 100 g of soil) without the use of hazardous substances is needed.

The union of sophisticated molecular techniques with classical soil sampling to quantify soil DNA is exemplified by the work of the South Australian Research & Development Institute (SARDI) in developing a DNA-based soil testing service. This soil test quantifies inoculum densities for several soilborne fungal and nematode pathogens including the false Columbia RKN (*M. fallax)*, the cereal cyst nematode (*Heterodera avenae*), and root-lesion nematodes (*P. neglectus* and *P. thornei*) (Ophel-Keller et al. 2008). Enhancing the recovery of pathogen DNA from soil was essential to the SARDI quantification approach. SARDI's method of DNA extraction directly from soil and quantification using qPCR was successful in quantifying DNA and placing fields into risk categories, giving support to the hypothesis that soil DNA levels determined before planting may provide an accurate prediction of damage and disease at harvest. However, the methods employed by SARDI are proprietary, necessitating the

development of new protocols and models for quantifying *M. hapla* in New York State potato production.

Spatial patterns and sampling strategies

Individual organisms occupying a regionalized area may assume certain patterns within space based on their biology or the influence of environmental factors. Spatial arrangements may be (i) aggregated or clustered in which individuals occur close together in space; (ii) uniform or regular, in which individuals are positioned more or less evenly over the area of interest; or (iii) random in which individuals occur at locations with no discernible pattern. The type of pattern is determined by the point intensity, that is, the average number of individuals occurring per unit area of the space (Illian et al. 2008). Plant-parasitic nematodes are spatially variable within the soil (Robertson and Freckman 1995), but are typically aggregated (Goodell and Ferris 1980; Campbell and Noe 1985). For example, populations of *G. rostochiensis*, *M. hapla*, *P. penetrans*, *H. avenae*, *H. trifolii*, and *Rotylenchulus reniformis* have been quantified as spatially aggregated within agricultural fields (Webster and Boag 1992; Wheeler et al. 1994; Wallace and Hawkins 1994; Farias et al. 2002).

Yet even with this knowledge, there remains a significant lack of data and critical literature regarding the spatiotemporal patterns of plant-parasitic nematodes (Robertson and Freckman 1995; Ettema et al. 1998). Knowledge of the spatiotemporal characteristics of soilborne epidemics may improve yield loss models, promote enhanced sampling strategies for pathogen monitoring, assist in selecting appropriate management tactics (including potential for site-specific pesticide application), and is important for understanding pathogen introduction events and the progress of the epidemic (Campbell and Noe 1985; Wheeler et al. 1994; Pethybridge et al. 2005). However, because of logistical and economic constraints, every location within an area

of interest cannot be sampled. Therefore, regionalized variables must be estimated over the area of interest from a fewer number of samples with known values.

A subset of spatial analysis known as geostatistics provides an approach for assessing spatial patterns of regionalized variables and determining the degree of spatial dependence (Cressie 1989; Chilès and Delfiner 2009). This technique was originally developed for the mining industry to predict spatial variability in the density of ore deposits (Matheron 1963). However, it has become a powerful tool within plant pathology and other biological sciences. Geostatistical analysis may be applied to data from regularly sampled areas to quantify and assess spatial patterning of organisms within fields, and predict values at non-sampled locations (Cressie 1989; Nelson et al. 1999).

Potato cultivar susceptibility

Predicting risk of crop damage may also be improved with knowledge of potato cultivar susceptibility to *M. hapla*. There is no commercially available genetic resistance in potato to *M. hapla* (Brown et al. 1994; Janssen et al. 1995; Melakeberhan et al. 2012), but some studies have noted differences in susceptibility between potato cultivars, measured by yield, plant vigor, and nematode reproductive measures (Van der Beek et al. 1998; Melakeberhan et al. 2012; Kandouh and Sipes 2014). In 2003, a study in New York State assessed 12 commercial potato cultivars for response to *M. hapla*, and found significant differences in RKN reproduction rates (Abawi et al. 2008). These studies used potato cultivars no longer favored by growers and limited additional work has been conducted. A recrudesce of investigation into the susceptibility of more recently introduced potato cultivars to *M. hapla* would aid potato growers, crop consultants, and university extension specialists in cultivar selection, and support more robust estimates of risk for *M. hapla* in potato.

Thesis Overview

The goal of this research was to quantify the risk of yield loss and crop damage in potato in New York State due to *M. hapla* by relating pre-plant population densities to observed yield loss and crop damage, with the outcome of supporting risk prediction and informed decision making for management. The specific objectives of this foundational research were:

1. To develop an efficient and economical method for extracting high quality nematode DNA from large volumes of soil (i.e. 100 g) for use in quantification experiments;
2. To develop a quantitative PCR method for quantifying *M. hapla* population densities using the RKN effector gene, *16D10*;
3. To determine the spatiotemporal patterns of *M. hapla* within commercial potato fields;
4. Evaluate damage thresholds for *M. hapla* in potato to determine if improvements may be made over standard manual counting methods using molecular-based methods; and
5. To determine the susceptibility of two commercially grown potato cultivars (cvs. Lamoka and Eva) to *M. hapla*.

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CHAPTER 2

ISOLATION OF NEMATODE DNA FROM 100 G OF SOIL USING Fe_3O_4 SUPER PARAMAGNETIC NANOPARTICLES*

Abstract

An economical method for extracting nematode DNA from 100 g of soil was developed to facilitate nematode detection and quantification, and tested using the Northern root-knot nematode, *Meloidogyne hapla*. The method utilized enzymatic laundry detergent lysis, Fe_3O_4 super paramagnetic iron oxide nanoparticle (SPION) capture, and polyvinylpolypyrrolidone (PVPP) purification. Resultant DNA from this SPION capture method was approximately 100-fold less but of similar quality to DNA obtained from a standard phenol procedure and a commercial DNA extraction kit. An addition of 10 mg of nanoparticles to the extraction lysate was identified to maximize DNA yield while minimizing co-capture of contaminants. The detection limit of the SPION capture method was approximately 100 nematodes per 100 g soil. The SPION capture method extracted nematode DNA from mineral soils but requires further optimization for extraction from high organic matter (i.e. 'muck') soils. Benefits of this method compared to alternative techniques are discussed.

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Introduction

Plant-parasitic nematodes are soilborne plant pathogens that cause significant crop loss through direct damage or facilitating entry by other organisms. The severity of damage and disease caused by plant-parasitic nematodes generally increases with population density (Barker and Olthof 1976; Olthof and Potter 1977; Fiers et al. 2012), but may also be affected by edaphic factors, environmental conditions, and host tolerance (Nicol et al. 2011).

Nematodes are often controlled with fumigant or non-fumigant nematicides that are applied to the soil prior to planting (Mitkowski and Abawi 2003). Quantification of plant-parasitic nematode populations in soil prior to planting of a crop promotes informed pesticide management decisions by verifying the presence of the pathogen and determining if populations are sufficient to warrant an application based on defined damage thresholds (Min et al. 2012; Hay et al. 2016). Predicting risk of crop damage from soilborne plant-parasitic nematodes is therefore strongly reliant upon effectively quantifying the presence of inoculum within a field (Madden et al. 2007) and the quantitative relationship between populations and yield of an annual crop (Barker and Olthof 1976).

Traditional methods of quantifying nematode populations in soil involve extraction of motile nematodes using active or passive collection techniques (McSorley and Walter 1991), then identification and counting under high power magnification (400×). Commonly used apparatus for active extraction of motile nematodes include Baermann funnels (Baermann 1917) and Whitehead trays (Whitehead and Hemming 1965). However, these techniques have substantial limitations in their ability to provide accurate estimations of populations because of low extraction efficiencies. These methods may also considerably underestimate populations due to the ability to extract only motile life stages (Hay et al. 2016). An additional limitation is an incubation period

of several days, leading to a substantial time lag in providing a sample suitable for enumeration. Passive extraction techniques such as sugar density gradients and Seinhorst's elutriator (Seinhorst 1965) require considerable time and investment in equipment. Populations enumerated using either type of technique are often counted based only on an aliquot of the sample, potentially reducing the accuracy and precision of counting. An additional shortcoming to morphological identification of nematodes is that at the magnifications used for enumeration, identification is often only possible to the genus level which may be suboptimal for making determinations on disease risk and whether control tactics are justified (Min et al. 2012). The process is also technically challenging and subject to operator fatigue.

Extraction of DNA from soil for enumerating plant-parasitic nematode population densities using molecular techniques has the potential to provide substantial improvements through improved sensitivity and specificity compared to traditional quantification techniques (Min et al. 2012). Hay et al. (2016) found that potato tuber damage caused by *Meloidogyne* spp. in Australia was accurately predicted by pathogen DNA quantification from soil prior to planting. Similarly, Stirling et al. (2004) reported that population estimates of *Meloidogyne* spp. obtained from DNA in soil were similar to those obtained through Whitehead trays, and improved upon the trays by facilitating automation and detection of multiple target organisms. For accurate detection, quantification, and risk assessment of soilborne pathogens by molecular analysis, a robust and reliable method for DNA extraction from soil is required.

Critical to accurate detection and quantification of DNA using molecular techniques is achieving complete tissue digestion and release of DNA into solution ('extraction' of DNA), and the precipitation of DNA from solution in the absence of contaminants, such as humic substances, phenolic compounds, polysaccharides, and proteins ('isolation' of DNA) (LaMontagne et al. 2002; Zhang et al. 2009; Sidstedt et al. 2015). For

quantification of nematode populations, one potential barrier to accurate enumeration lies in incomplete tissue digestion and release into solution because of resistance of the nematode cuticle to commonly used lysis chemicals, including sodium dodecyl sulfate, due to the presence of cross-linked collagens, keratin and other proteins in the cuticle (Bird and Bird 1991; Johnstone 1994). In circumventing this challenge, numerous protocols for DNA extraction from soil have been described (e.g., Steffan et al. 1988; Purdy et al. 1996; Yan et al. 2008; Kathiravan et al. 2015). These protocols have produced high quality DNA but their adoption is limited by the time required, technical challenges required for optimization, and need to use phenol or chloroform, rendering them hazardous to the operator and environment (Bandyopadhyay et al. 2011). Commercial DNA extraction kits offer an alternative, which are generally easy to use and do not produce hazardous chemical waste. However, many of these kits are cost prohibitive and are limited to processing small volumes of soil (often less than 10 g) which restricts the ability for the results to be translated to field level management recommendations for soilborne plant pathogens (Hay et al. 2016). McSorley and Walter (1991) indicated that soilborne organisms, including plant-parasitic nematodes are spatially aggregated and a high frequency of soil samples need to be taken and bulked, with a subsample then removed for analysis. To illustrate, Herdina and Roget (2000) showed the accuracy of quantification of the soilborne wheat root-rot pathogen *Gaeumannomyces graminis* was increased when larger quantities of soil (50 to 250 g of soil) were processed to overcome the spatial heterogeneity of the pathogen within a field. Thus, an ideal extraction method would use at least 50 g of soil. Alternative physical methods for DNA extraction have included bead beating (Yan et al. 2008) and compaction (Goto et al. 2009), and mill rotor grinding (Brierley et al. 2009). These methods often use specialized equipment making initial fixed costs high and in many cases are a substantial barrier to adoption. Stirling et al. (2004) and Hay et al. (2016)

used the DNA extraction method developed by the South Australian Research & Development Institute for pathogen detection. This method is capable of isolating nematode DNA from 500 g soil samples but is proprietary (Ophel-Keller et al. 2008).

Magnetic bioseparation using super paramagnetic iron oxide nanoparticles (SPION) has recently gained popularity, and offers a rapid and economical DNA isolation method that captures DNA on solid nanoparticle supports through reversible binding (Tiwari et al. 2015). A substantial advantage of SPION methods for DNA extraction is elimination of the requirement to use ethanol or isopropanol, which may exacerbate the co-precipitation of contaminants and disrupt downstream applications, such as PCR (Arbeli and Fuentes 2007). SPION-based isolation protocols have been described for extracting genomic DNA using Fe_3O_4 SPION from laboratory grown microbial cultures (Bandyopadhyay et al. 2011), biological tissues (Tiwari et al. 2015) and environmental soil samples (Sebastinanelli et al. 2008; Paul et al. 2014). Although these isolation protocols from soil have been described for small volumes (approximately 0.5 g of soil), the method has potential for processing increased soil volumes, to negate trade-offs between DNA extraction from soil for pathogen quantification and inherent spatial heterogeneity within fields (Campbell and Noe 1985; Wheeler et al. 1994; King and Taberna 2013).

The objective of this study was to develop and evaluate a high-throughput method for DNA extraction from large (100 g) soil samples as a precursor for the detection and quantification of plant-parasitic nematodes for informing management decisions by growers. The Northern root-knot nematode (RKN), *Meloidogyne hapla*, was used as the model organism for this study. *M. hapla* is a sedentary endoparasite of potato and numerous other temperate vegetable crops worldwide (Olthof and Potter 1972; Mitkowski and Abawi 2003; Bridge and Starr 2007). In the temperate production area of the Northeastern United States, *M. hapla* is the most common species (Walters and

Barker 1994) and causes reduced yields, damage and deformities to belowground structures, and may predispose roots to infection by other soilborne fungi (Mitkowski et al. 2002). Adult females, and third- and fourth-stage juveniles may be found within the plant roots, while eggs, second-stage juveniles (J2s), and adult males are found in the soil (Bridge and Starr 2007).

Materials and Methods

***Meloidogyne hapla* inoculum and inoculation of soil samples.** Soil samples were prepared by placing 0.5 g of a pasteurized soil mix (three parts top-soil to one part sand) in a 2 ml tube which were inoculated with 10 *M. hapla* J2s, hereafter referred to as *M. hapla*. Inoculum was prepared by rearing nematodes hydroponically on a RKN susceptible tomato cultivar (cv. Rutgers; Appendix A). The hydroponics system was constructed as follows: tomato plants infected with *M. hapla* were grown for 30 days on sterilized sand. Sand was then washed with tap water from roots and plants with bare roots were placed into hydroponics tanks (GroMomma Bubbler Bucket, Sunlight Supply Inc., Vancouver, WA). Plants were grown for 15 days, maintaining reservoir water at a pH of 6.0. *M. hapla* J2s were collected by passing reservoir water over a 25 micron mesh sieve held at a 45° angle. The solution was then subsequently passed through 75 and 45 micron mesh sieves to remove excess debris. The number of *M. hapla* within the solution was counted at 400× and the appropriate volume of inoculum applied. Inoculated soil samples were air dried at room temperature for 10 days and randomly assigned to treatments.

Comparison of extraction methods. DNA extraction from 0.5 g soil inoculated with 10 *M. hapla* J2s each were compared using a SPION capture method, a standard phenol extraction, and a commercial DNA isolation kit. Five replicate soil samples were used

for each method and the experiment was conducted twice. Negative extraction controls (0.5 g sterilized soil without *M. hapla*) were included for each method.

SPION capture method. One ml of Lysis Buffer (100 mM Tris hydrochloride, 100 mM EDTA, 10 mM sodium chloride, 3% w/v enzymatic laundry detergent (Tide Original Powder Laundry Detergent, Procter and Gamble, Cincinnati, OH), and 10 mg/ml RNase A (Qiagen Inc., Carlsbad, CA), buffer adjusted to pH 10.4) was added to the soil sample and gently vortexed. Samples were incubated until the internal temperature of the solution reached 80°C for 5 min, and vortexed at maximum speed for 30 sec. Initial experiments assessing the requirement for laundry powder with reduced heating (50°C) did not produce DNA (data not shown), indicating that heating to 80°C was necessary. Samples were then centrifuged at 5000 *g* for 5 min, and the supernatant transferred to a new 2 ml tube. To this supernatant, 1 mg of SPION (Fe_3O_4 nanoparticles, 20 to 30 nm; SkySpring Nanomaterials Inc., Houston, TX; suspended in sterile distilled water) and 1 ml of Binding Buffer (20% w/v polyethylene glycol 8000, 4 M NaCl) were added. The SPION were sonicated immediately prior to use for 10 min using a microtip probe (Model SFX150, Branson Instruments, Danbury, CT) at 50% amplitude. The extraction mixture was incubated at room temperature for 20 min, with gentle inversion. SPION were then immobilized using a magnetic stand (New England Biolabs, Ipswich, MA) and supernatant was discarded. SPION were washed three times with 70% ethanol. Samples were dried in sterile conditions for 1 h to remove trace ethanol. DNA was eluted by washing 100 µl of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.3) over the SPION, and incubating at room temperature for 5 min. SPION were immobilized and resultant solution containing DNA transferred to a fresh microcentrifuge tube. Once isolated from solution, DNA must be purified for use in molecular analysis. Polyvinylpolypyrrolidone (PVPP) has been noted for its ability to bind humic acids and other contaminants from soil, and has been used in DNA

purification (Berthelet et al. 1996; LaMontagne et al. 2002; Kathiravan et al. 2015). Potential contaminants were removed from the crude DNA extraction with a PVPP spin column. The PVPP spin column was made by placing a 0.5 ml tube within a 2 ml tube. A sterile needle was used to make a hole in the bottom of the smaller tube and a small quantity of sterile glass wool was compacted into the end. PVPP (110 µm particle size, Sigma-Aldrich, St. Louis, MO) was suspended in TE buffer (pH 8.3) until a thick paste was formed and pipetted onto the glass wool support. The assembly was centrifuged at 900 *g* for 1.5 min to pack the column and remove excess buffer. The building and packing steps were repeated as necessary until the column was 15 mm high. The completed PVPP column was then nested into a new 2 ml tube, and the crude DNA extract pipetted on top of the column. The assembly was incubated for 10 min at room temperature, then centrifuged at 400 *g* for 1.5 min. Eluted DNA was stored at -80°C.

Standard phenol method. DNA was isolated using a standard phenol method as described by Purdy et al. (1996), with minor modifications described herein. In brief, 700 µl lysis buffer (120 mM sodium phosphate (pH 8.0), 1% w/v acid washed PVPP), 50 µl 20% w/v SDS, and 500 µl phenol were added to soil samples. Samples were shaken three times at 30 Hz for 30 sec on a Qiagen TissueLyser System, with 30 sec on ice between shaking steps. Samples were centrifuged at 12,000 *g* for 2 min, and supernatant transferred to a new microcentrifuge tube. A hydroxyapatite (HTP; Bio-Rad Laboratories, Inc., Hercules, CA) spin column was made by nesting a 0.5 ml microcentrifuge tube inside a 2 ml tube. A hole was made in the bottom of the smaller tube, and a small quantity of sterile glass wool compacted into the end. Approximately 300 µl of hydrated HTP was pipetted on top of the glass wool. Crude DNA extract was placed on top of the HTP spin columns and centrifuged at 100 *g* for 2 min. Columns were washed three times with 500 µl of 120 mM sodium phosphate (pH 7.2). Nucleic acids were eluted from the column with 400 µl of 300 mM potassium phosphate (pH

7.2), and desalted using a DNA Clean & Concentrator kit (Zymo Research Corp., Invine, CA). Resultant DNA was suspended in 100 µl of sterile TE buffer and stored at -80°C.

Commercial kit method. DNA was isolated from 0.5 g soil using the MO BIO PowerLyser PowerSoil DNA extraction kit (Qiagen Inc.) according to the manufacturer's instructions. Resultant DNA was suspended in 100 µl of sterile TE buffer and stored at -80°C.

Binding capacity of the SPION capture method on 100 g of soil. To quantify the binding capacity of 20 to 30 nm nanoparticles within the SPION capture method, resultant DNA yield was quantified after using increasing quantities (1, 5, 10, 20 and 50 mg) of nanoparticles. Aliquots (100 g) of a pasteurized soil mix (three parts top-soil to one part sand) were inoculated with approximately 200 *M. hapla*. Five replicate extractions were conducted for each quantity of nanoparticles, and the experiment conducted twice. Negative extraction controls (100 g sterilized soil without *M. hapla*) were assessed using 5 mg SPION.

The 100 g-SPION capture method was performed by placing soil samples into a 250 ml Nalgene bottle with screw cap lid and 50 ml of Lysis Buffer followed by gentle vortexing to mix. Samples were heated in a water bath until the temperature of the solution reached 80°C for 5 min, after which they were vortexed at maximum speed for 30 sec on a VortexGenie fitted with a flat top attachment (VWR, Radnor, PA). Bottles were centrifuged at 5000 *g* for 5 min and supernatant transferred to a 50 ml falcon tube (supernatant measured ≤ 25 ml). To the supernatant, SPION and 25 ml of Binding Buffer were added. The mixture was then incubated at room temperature for 20 min, with gentle inversion. SPION were immobilized using a magnetic rack, and supernatant discarded. SPION were washed three times with 70% ethanol for the 1, 5, and 10 mg sets, and washed four times with 70% ethanol for the 20 and 50 mg sets. SPION were

dried in sterile conditions for 2 h to remove trace ethanol. DNA was then eluted from the particles by washing 100 µl of sterile TE buffer over the SPION, and incubating at room temperature for 5 min. SPION were immobilized with a magnetic rack and DNA solution transferred to a 1.5 ml tube. Crude DNA extract was purified using a PVPP spin column as described above, and resultant DNA was stored at -80°C.

Sensitivity of the SPION capture method using 100 g of soil. The sensitivity of the 100 g-SPION capture method, operationally defined here as the lowest number of nematodes detectable by amplification of a target sequence using PCR, was evaluated. Aliquots (100 g) of a pasteurized soil mix (three parts top-soil to one part sand) were inoculated with 1, 10, 100, or 1,000 *M. hapla*. For soil samples inoculated with one *M. hapla*, a single nematode was identified in the inoculum solution at 63× magnification, removed with an ultra-fine dental pick and transferred to the soil. The dental pick was then re-examined to ensure deposition of the nematode. The 100 g-SPION capture method was used with 10 mg nanoparticles to extract DNA from all samples. Five replicates of each *M. hapla* density were included, and the entire experiment was repeated twice. Negative extraction controls (100 g sterilized soil without *M. hapla*) were included. DNA was eluted by washing SPION with 100 µl of sterile TE buffer, and incubating at room temperature for 5 min. SPION were immobilized with a magnetic rack and DNA solution transferred to a 1.5 ml tube. Crude DNA extract was purified using a PVPP spin column as described above. Resultant DNA was stored at -80°C.

Robustness of DNA extraction with SPION from 100 g of soil across soil type. The utility of the optimized SPION capture method with 10 mg nanoparticles across different soil types was determined. Two mineral and two high organic matter muck soils were collected from vegetable fields in New York State (Mineral Soil 1: loam, Pavilion (N 42.88, W -78.02); Mineral Soil 2: sandy loam, Caledonia (N 42.98, W

-77.86); Muck Soil 1: Newark (N 43.04, W -77.09); Muck Soil 2: Marion (N 43.14, W -77.19)). Soil was sterilized by autoclaving three times at 100°C for 25 min over three consecutive days, then proportioned into 100 g aliquots. Two hundred *M. hapla* were inoculated from an inoculum solution into each aliquot, as described above. The 100 g-SPION capture method was performed as described above, using 10 mg of nanoparticles across all samples, with six replicates of each of the four soils. Negative extraction controls (100 g sterilized soil of each type without *M. hapla*) were included. DNA was eluted by washing SPION with 100 µl of sterile TE buffer, and incubating at room temperature for 5 min. SPION were immobilized with a magnetic rack and DNA solution transferred to a 1.5 ml tube. Crude DNA extract was purified using a PVPP spin column as described above, and resultant DNA was stored at -80°C.

Assessment of DNA quantity and quality. For each experiment, extracted genomic DNA was quantified and assessed for quality. Firstly, DNA degradation was visualized by separating crude DNA (15 µl) on a 1% agarose gel stained with 1× GelRed at 50 V for 70 min, with 15 ng of lambda DNA (New England Biolabs, 48 kb molecular weight) as a marker. Following PVPP purification, eluted DNA was quantified (ng) using a Qubit fluorimeter (Thermo Fisher Scientific, Waltham, MA) and assessed for quality ($A_{260/280}$ and $A_{260/230}$) using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

The suitability of each extraction method to produce DNA without inhibitors which may interfere with PCR was assessed by amplifying the rDNA internal transcribed spacer (ITS) region using *M. hapla* species-specific primers (Hay et al. 2016). Amplification of an approximately 150 bp size band is indicative of the presence of *M. hapla*. PCR reactions were performed in a total volume of 50 µl, containing 4 µl of DNA product, 200 µM of each primer, 200 µM dNTP, 1% bovine serum album, 1.25 U *Taq* polymerase, and 1× Standard *Taq* Buffer (New England BioLabs). Amplification conditions consisted of initial denaturation at 95°C for 5 min, followed by 38 cycles of

95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final elongation step of 72°C for 5 min, using a C1000 Touch programmable thermocycler (Bio-Rad Laboratories Inc.). PCR products were separated on a 1% agarose gel stained with 1× GelRed at 50 V for 120 min, and size was compared to a 100 bp ladder (Axygen Scientific, Corning Inc., Corning, NY). The percentage of samples in which the ITS region of *M. hapla* was successfully amplified was summarized.

Statistical analysis. Statistical analysis for the four individual experiments were completed in R (v. 3.3.3; R Core Team, 2014). To determine whether results from replicated trials could be combined, means were compared using a Wilcoxon rank sum test (Mann and Whitney 1947) and the homogeneity of variances assessed using Fisher's F-Test (Markowski and Markowski 1990) function in R package 'stats'. No evidence was found for significant differences in the mean or variances between experiments, so results of both experiments were combined for analysis. The effect of protocol, SPION volume, nematode inoculation quantity, and soil type on DNA yield (ng) were analyzed using generalized linear models, removing outliers with high leverage where necessary. Treatment means were separated using Least Significant Differences calculated using the R package 'agricolae'.

Results

Comparison of extraction methods. High molecular weight and good quality DNA was extracted from soil from using each of the three methods (Fig. 2.1A). Total DNA yield was 98.7% lower using the SPION capture method than the average of the commercial kit and standard phenol methods ($P < 0.0001$; Table 2.1; Fig. 2.2A). The phenol method produced DNA of significantly higher quality ($A_{260/280}$) than the other methods ($P = 0.019$; Table 2.1). In contrast, DNA from the commercial kit had significantly higher $A_{260/230}$ ratios than the other methods ($P < 0.0001$; Table 2.1; Fig.

2.2B, C). *Meloidogyne hapla* was detected by PCR amplification in 70, 50, and 90% of samples for the SPION capture method, phenol extraction, and commercial kit, respectively (Table 2.1; Fig. 2.1B). Amplification products were not observed in the negative extraction controls (Fig. 2.1B).

Binding capacity of the SPION capture method on 100 g of soil. Increasing the quantity of nanoparticles from 1 to 50 mg had no significant effect ($P = 0.692$) on DNA yield (Table 2.2; Fig. 2.3A). There was no significant difference in $A_{260/280}$ or $A_{260/230}$ ratios between any of the SPION volume groups ($P = 0.077$ and 0.541, respectively; Table 2.2). The percent of samples detectable by PCR for *Meloidogyne* spp. were 70, 80, 80, 30, and 30%, respectively, indicating a decrease in functional quality and suitability for molecular downstream applications when using 20 and 50 mg nanoparticles (Table 2.2; Fig. 2.3B). No amplification products were observed in the negative extraction controls (Fig. 2.3B).

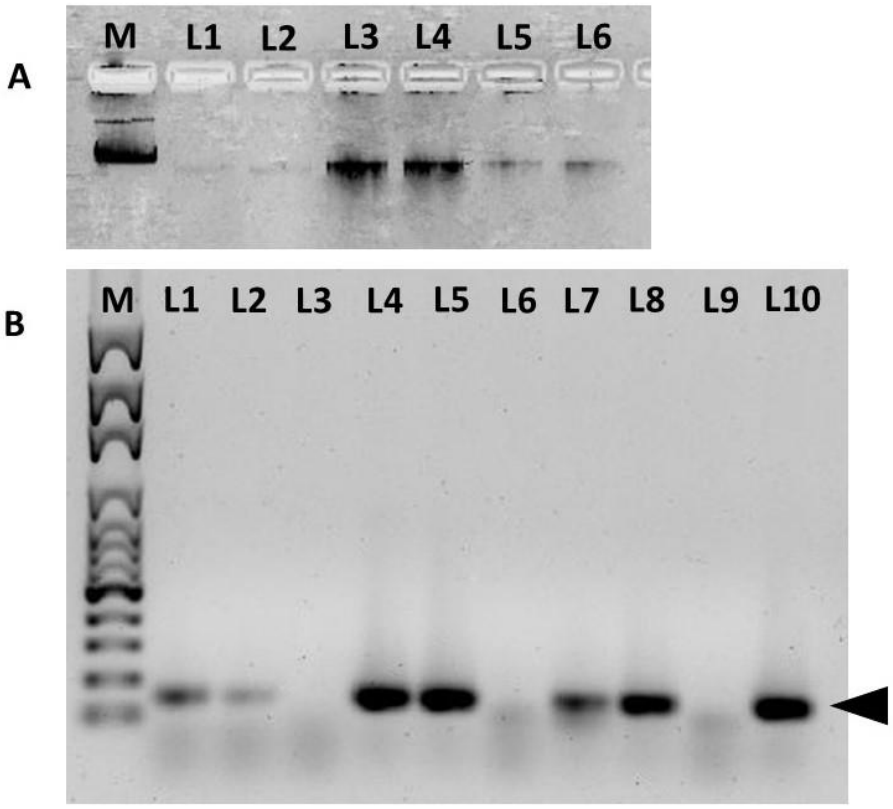

Fig. 2.1. Genomic DNA isolated using each extraction method visualized on an agarose gel stained with GelRed (**A**). M: 15 ng phage lambda DNA, molecular weight 48 kb. Lanes 1 and 2: SPION capture method. Lanes 3 and 4: conventional phenol method. Lanes 5 and 6: commercial kit extraction. Resultant DNA from each method was PCR amplified using *Meloidogyne hapla* species-specific primers (**B**; Hay et al. 2016) targeting a segment of the internal transcribed spacer (ITS) region. Amplification products are approximately 150 bp in size (arrow-head). M: 100 bp DNA ladder. Lanes 1 and 2: SPION capture method. Lane 3: SPION capture method negative control. Lanes 4 and 5: conventional phenol method. Lane 6: phenol method negative control. Lane 7 and 8: commercial kit extraction. Lane 9: commercial kit extraction negative control. Lane 10: positive PCR control amplified from *M. hapla*.

Table 2.1. A Fe_3O_4 super paramagnetic iron oxide nanoparticle (SPION) based genomic DNA extraction from soil method was compared to a standard phenol-based method and a commercial kit. Approximately 10 *Meloidogyne hapla* second-stage juveniles were inoculated to 0.5 g of soil, and then air dried before performing the extraction. Resultant DNA was assessed for quantity (pg/µl) and quality ($A_{260/280}$ and $A_{260/230}$ ratios, and percent of samples detectable by PCR). Five replicates of each method were performed and the experiment was repeated twice, with results averaged across all replicates. Means followed by the same letter within columns are not significantly different at the 0.05 level.

Protocol	**DNA yield (pg/µl)**	**$A_{260/280}$[a]**	**$A_{260/230}$[b]**	**Detectable by PCR (%)**
SPION	32.6 b	1.60 a	0.25 b	70
Phenol	3.06×10^5 a	1.84 b	0.37 b	50
Kit	1.99×10^5 a	1.56 a	0.97 a	90
LSD[c]	1127	0.26	0.25	
F =	15.6	4.62	18.7	
P =	< 0.0001	0.019	< 0.0001	
CV (%)[d]	SPION: 70.6 Phenol: 66.5 Kit: 30.9	SPION: 6.46 Phenol: 23.8 Kit: 14.7	SPION: 11.7 Phenol: 87.1 Kit: 37.7	

[a] The ratio of spectral absorbance at 260 and 280 nm ($A_{260/280}$) is a quantitative measure of protein contamination in a nucleic acid sample. A ratio of 1.80 represents a sample without protein contamination.

[b] The ratio of spectral absorbance at 260 and 230 nm ($A_{260/230}$) is a quantitative measure of residual salt contamination. Higher values indicate less residual salts.

[c] LSD, least significant difference.

[d] CV, coefficient of variation is the ratio of the standard deviation to the mean, quantifying dispersion of the data.

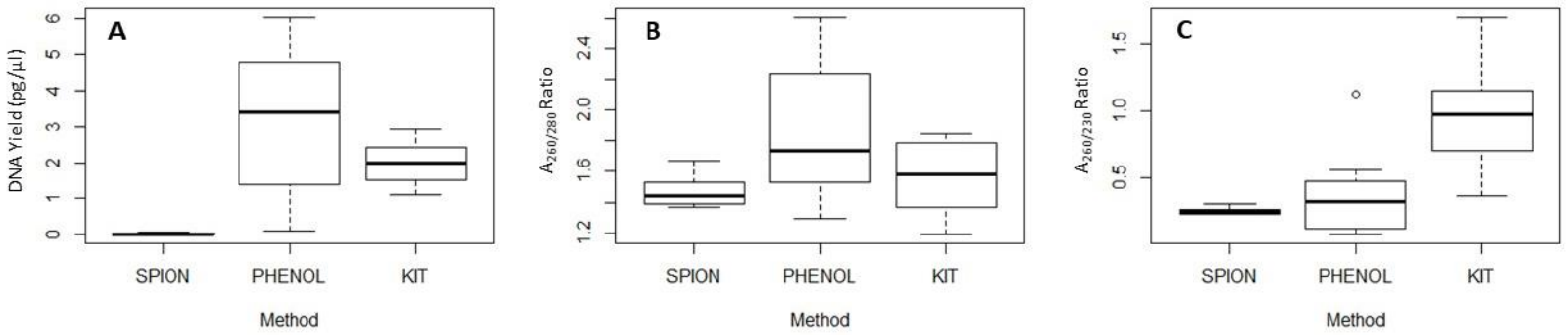

Fig. 2.2. A super paramagnetic iron oxide nanoparticle (SPION) based DNA extraction method was performed on 0.5 g of soil and compared to a standard phenol method and a commercial kit. Each soil sample was inoculated with 10 *Meloidogyne hapla* second-stage juveniles from an inoculum solution and air dried. Ten replicates of each method were performed and the experiment was repeated. Box-plots were generated from the data to evaluate variability in the DNA yield (pg/µl) (**A**), $A_{260/280}$ ratio (**B**), and $A_{260/230}$ ratio (**C**). Within each plot, the median is represented by the bold line. Upper and lower quartiles are represented by the edges of the box. Box whiskers denote maximum and minimum values, and dots mark outliers.

Table 2.2. DNA extraction from soil using a super paramagnetic iron oxide nanoparticle (SPION) capture method was assessed for the binding capacity of 20 to 30 nm Fe_3O_4 nanoparticles. One hundred gram samples of sterilized soil were inoculated with 200 *Meloidogyne hapla* second-stage juveniles from an inoculum solution and air dried. The method was performed as described in the text, with adding variable masses of nanoparticles, 1, 5, 10, 20, and 50 mg, and assessed for quantity (pg/µl) and quality ($A_{260/280}$ and $A_{260/230}$ ratios, and percent of samples detectable by PCR). Five replicates of each nematode level were performed and the experiment was repeated twice, with results averaged across all replicates. Ten mg of nanoparticles gave the highest DNA yield, however the result was not significantly different between nanoparticle masses. The 20 and 50 mg sets had greatly reduced PCR amplification, suggesting increased co-extraction of contaminants.

SPION volume (mg)	**DNA yield (pg/µl)**	**$A_{260/280}$[a]**	**$A_{260/230}$[b]**	**Detectable by PCR (%)**
1	49.9	1.64	0.23	70
5	42.9	1.52	0.27	80
10	59.3	1.58	0.26	80
20	21.7	1.65	0.26	30
50	32.1	1.70	0.27	30
LSD[c]	54.9	0.13	0.07	
F =	0.561	2.26	0.786	
P =	0.692 (ns)	0.077 (ns)	0.541 (ns)	

[a] The ratio of spectral absorbance at 260 and 280 nm ($A_{260/280}$) is a quantitative measure of protein contamination in a nucleic acid sample. A ratio of 1.80 is considered free of protein contaminants.

[b] The ratio of spectral absorbance at 260 and 230 nm ($A_{260/230}$) is a quantitative measure of residual salt contamination. Higher values indicate less residual salts.

[c] LSD, least significant difference.

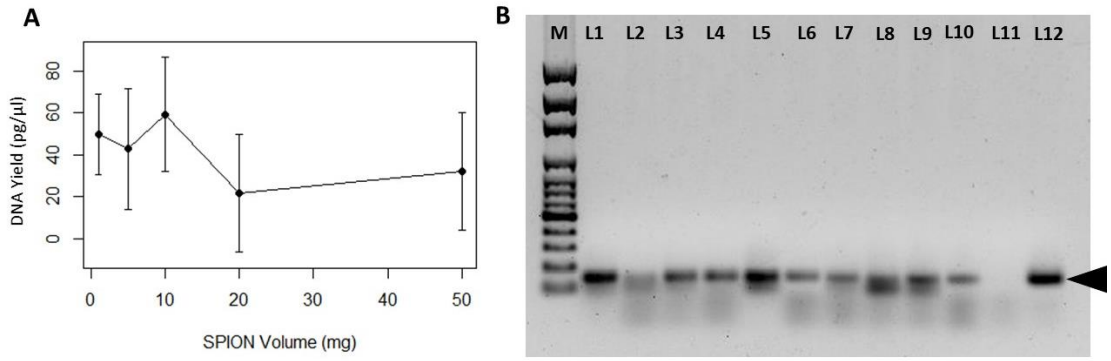

Fig. 2.3. Addition of increasing amounts of super paramagnetic iron oxide nanoparticles (SPION) from 1 to 50 mg had no significant effect on DNA yield (**A**). Bars in the Fig. represent the standard error of the mean. Resultant DNA from each SPION volume trial was PCR amplified using *Meloidogyne hapla* species-specific primers (**B**; Hay et al. 2016). Amplification products are approximately 150 bp in size (arrow-head). Lanes 1 and 2: 1 mg SPION. Lanes 3 and 4: 5 mg SPION. Lanes 5 and 6: 10 mg SPION. Lanes 7 and 8: 20 mg SPION. Lanes 9 and 10: 50 mg SPION. Lane 11: extraction negative control. Lane 12: positive PCR control amplified from *M. hapla*. M: 100 bp DNA ladder. Consistent amplification is noted at the addition of 10 mg of nanoparticles.

Sensitivity of the SPION capture method using 100 g of soil. Increasing the number of nematodes within 100 g soil resulted in a significant increase in DNA yield. Soil samples inoculated with 1,000 *M. hapla* J2s had significantly higher DNA yield than those inoculated with 1, 10, or 100 individuals (P = 0.016; Table 2.3; Fig. 2.4A). Nematode numbers had no significant difference in $A_{260/280}$ or $A_{260/230}$ ratios (P = 0.059 and 0.788, respectively; Table 2.3). Soil samples inoculated with 100 and 1,000 *M. hapla* produced visible, high molecular weight bands (Lanes 5 to 8 in Fig. 2.4B). Bands were not observed from DNA extracted from soil samples with 1 and 10 *M. hapla* (Lanes 1 to 4 in Fig. 2.4B). The percentage of samples in which *M. hapla* was detected by PCR was 20, 40, 90, and 90% for the soil samples inoculated with 1, 10, 100 and 1,000 *M. hapla*, respectively (Table 2.3; Fig. 2.5) and no amplification products were observed in negative extraction controls (Fig. 2.5).

Table 2.3. The 100 g- super paramagnetic iron oxide nanoparticle (SPION) capture method was assessed for sensitivity. Soil samples of 100 g were sterilized and inoculated with 1, 10, 10, or 1,000 *Meloidogyne hapla* second-stage juveniles and air dried. The method was performed as described in the text for the 100 g-SPION capture method, and assessed for quantity (pg/µl) and quality ($A_{260/280}$ and $A_{260/230}$ ratios, and percent of samples detectable by PCR). Five replicates of each nematode level were performed and the experiment was repeated twice, with results averaged across all replicates. DNA yield increased as nematode inoculation number increased, with 1,000 nematodes producing significantly more DNA than the 1, 10, or 100 nematode groups. Means followed by the same letter within columns are not significantly different at the 0.05 level.

Nematodes per 100 g soil	**DNA yield (pg/µl)**	**$A_{260/280}$[a]**	**$A_{260/230}$[b]**	**Detectable by PCR (%)**
1	7.40 a	1.65	0.29	20
10	22.0 ab	1.61	0.33	40
100	30.0 ab	1.53	0.30	90
1,000	156.0 b	1.58	0.36	90
LSD[c]	118	0.12	0.08	
F =	6.30	1.63	1.37	
P =	0.016	0.589 (ns)	0.268 (ns)	
CV (%)[d]	One: 66.1	One: 5.94	One: 17.9	
	Ten: 92.6	Ten: 7.78	Ten: 30.3	
	Hundred: 123.9	Hundred: 11.6	Hundred: 7.11	
	Thousand: 177.8	Thousand: 6.87	Thousand: 35.4	

[a] The ratio of spectral absorbance at 260 and 280 nm ($A_{260/280}$) is a quantitative measure of protein contamination in a nucleic acid sample. A ratio of 1.80 is considered free of protein contaminants.

[b] The ratio of spectral absorbance at 260 and 230 nm ($A_{260/230}$) is a quantitative measure of residual salt contamination. Higher values indicate less residual salts.

[c] LSD, least significant difference.

[d] CV, coefficient of variation is the ratio of the standard deviation to the mean, quantifying dispersion of the data.

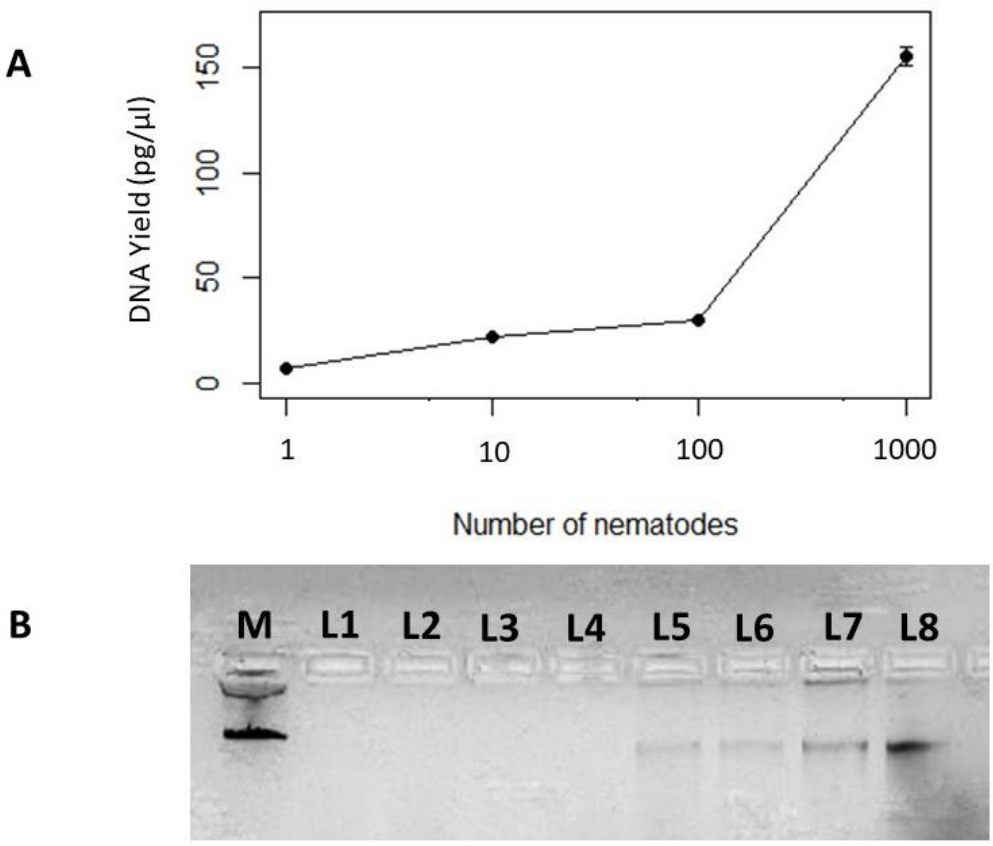

Fig. 2.4. Increasing the number of inoculated nematodes resulted in an increasing DNA yield isolated using 100 g-SPION capture method from soil samples inoculated with 1, 10, 100, or 1,000 *Meloidogyne hapla* second stage juveniles (**A**). However, only the 1,000 nematodes per 100 g soil was significantly greater than the population densities. Bars represent standard error of the mean. Resultant genomic DNA (15 µl) was separated on a 1% agarose gel stained with GelRed (**B**). Lanes 1 and 2: 1 nematode per 100 g soil. Lanes 3 and 4: 10 nematodes per 100 g soil. Lanes 5 and 6: 100 nematodes per 100 g soil. Lanes 7 and 8: 1,000 nematodes per 100 g soil. M: 15 ng phage lambda DNA.

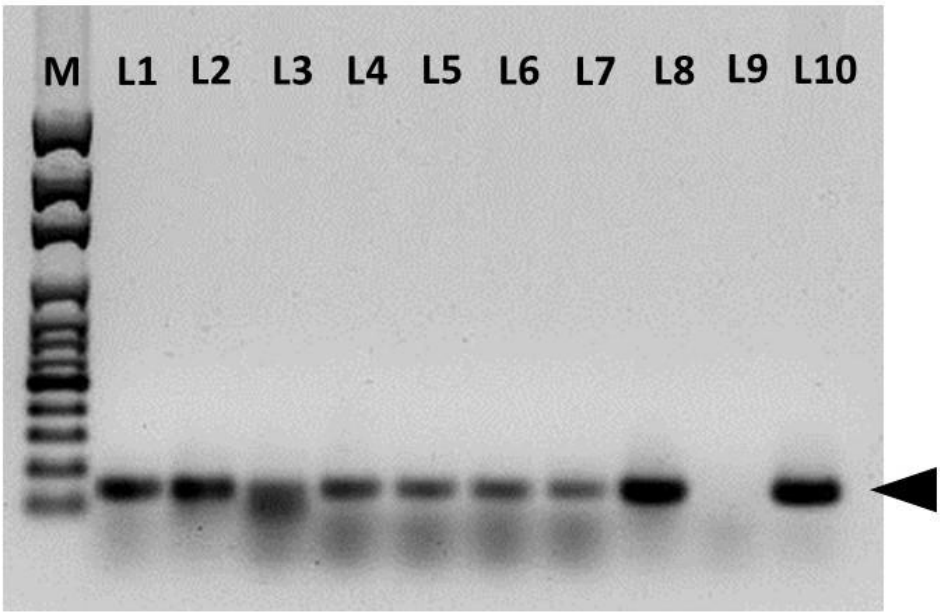

Fig. 2.5. Resultant DNA from sensitivity testing of the 100 g-SPION capture method was PCR amplified using *Meloidogyne hapla* species-specific primers (Hay et al. 2016). Products are approximately 150-bp in size (arrow-head). Lanes 1 and 2: 1 nematode per 100 g soil. Lanes 3 and 4: 10 nematodes per 100 g soil. Lanes 5 and 6: 100 nematodes per 100 g soil. Lanes 7 and 8: 1,000 nematodes per 100 g soil. Lane 9: extraction negative control. Lane 10: positive PCR control amplified from *M. hapla*. M: 100 bp DNA ladder.

Robustness of DNA extraction with SPION from 100 g of soil across soil type. The two mineral soils had an average DNA yield of 23.5 and 60.7 pg/µl, and there was no significant difference between the quantity ($P = 0.207$) or quality ($A_{260/280}$, $P = 0.232$; $A_{260/230}$, $P = 0.551$) measurements (Table 2.4). PCR products indicative of *M. hapla* were amplified in 11 of the 12 extracted from mineral soil samples (Fig. 2.6). No amplification products were observed in the negative extraction controls (Fig. 2.6). No DNA was extracted from the muck soil samples, due to insufficient supernatant from the centrifugation step.

Table 2.4. The 100 g- super paramagnetic iron oxide nanoparticle (SPION) capture method was assessed for its utility on different soil types. Two mineral soils and two muck soils were inoculated with 200 *Meloidogyne hapla* second-stage juveniles per sample, and air dried. DNA was isolated as described for 100 g in the text, and assessed for quantity (pg/µl) and quality ($A_{260/280}$ and $A_{260/230}$ ratios, and percent of samples detectable by PCR). Six replicates per soil type were processed. The method produced high quality DNA in both mineral soil samples. The muck soils did not yield any DNA due to insufficient supernatant obtained during the lysis step.

Soil type	**DNA yield (pg/µl)**	**$A_{260/280}$[a]**	**$A_{260/230}$[b]**	**Detectable by PCR (%)**
Mineral Soil 1	23.5	1.69	0.28	83
Mineral Soil 2	60.7	1.76	0.27	100
Muck Soil 1	No yield	-	-	-
Muck Soil 2	No yield	-	-	-
LSD[c]	61.5	0.12	0.03	
F =	1.82	1.62	0.38	
P =	0.207 (ns)	0.232 (ns)	0.551 (ns)	
CV (%)[d]	Mineral 1: 96.1 Mineral 2: 105.0	Mineral 1: 3.71 Mineral 2: 6.59	Mineral 1: 6.8 Mineral 2: 10.2	

[a] The ratio of spectral absorbance at 260 and 280 nm ($A_{260/280}$) is a quantitative measure of protein contamination in a nucleic acid sample. A ratio of 1.80 is considered free of protein contaminants.

[b] The ratio of spectral absorbance at 260 and 230 nm ($A_{260/230}$) is a quantitative measure of residual salt contamination. Higher values indicate less residual salts.

[c] LSD, least significant difference.

[d] CV, coefficient of variation is the ratio of the standard deviation to the mean, quantifying dispersion of the data.

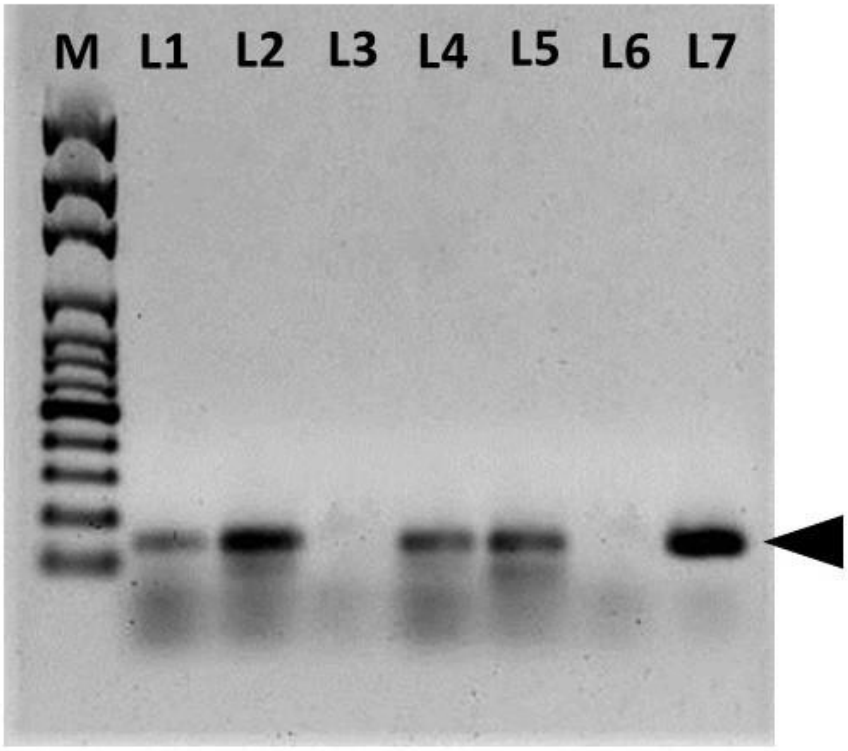

Fig. 2.6. Resultant DNA from the 100 g-SPION capture method performed with two mineral soil samples was PCR amplified using *Meloidogyne hapla* species-specific primers (Hay et al. 2016). Products are approximately 150-bp in size (arrow-head). Lanes 1 and 2: two representative samples from Mineral Soil 1. Lane 3: negative extraction control from Mineral Soil 1. Lanes 4 and 5: two representative samples from Mineral Soil 2. Lane 6: negative extraction control from Mineral Soil 2. Lane 7: positive PCR control amplified from *M. hapla*. M: 100 bp DNA ladder.

Discussion

An economical method for extracting high quality DNA from large (100 g) volumes of soil, using enzymatic laundry powder lysis, SPION capture, and PVPP purification was developed to facilitate DNA-based enumeration of plant-parasitic nematodes. Enzymatic laundry powder was used in tissue lysis as an economical source of proteases, lipases, and surfactants. In a preliminary study of the suitability of the laundry powder, two solutions of 60 *M. hapla* second-stage juveniles each were prepared, and to one, 3% w/v laundry detergent powder was added. Both solutions were incubated at

80°C for 30 min. In the solution containing the laundry powder, approximately 66% of the nematodes had degraded, while remaining nematodes appeared shriveled, and interior body parts were unrecognizable. Nematodes in the solution without laundry powder appeared plump, with interior body components defined. The benefits of including enzymatic laundry powder for tissue lysis has been demonstrated on animal tissues (Bahl and Pfenninger 1996) and hair shafts (Guan et al. 2013), which contain keratin, collagens, and other proteins. Other studies have also used collagenases to disrupt the nematode cuticle and increase DNA yields (Martin et al. 2009). In this study, the combination of enzymatic laundry powder and elevated heat were capable of lysing the nematode cuticle and releasing DNA for isolation. Commercially available enzymatic laundry powder was an economical lysis material for DNA extraction from nematodes.

The high surface area to volume ratio that is characteristic of nanoparticles is an important benefit of this method for efficient capture of DNA (Tiwari et al. 2015). In studies conducted by Sebastinanelli et al. (2008), Bandyopadhyay et al. (2011), and Paul et al. (2014), nanoparticles ranged in size between 8 and 10 nm. For equal masses, the nanoparticles used in this study (20 to 30 nm) had a smaller total surface area compared to the smaller diameter nanoparticles. Here it was shown that the larger nanoparticles were also capable of reversibly binding and efficient separation of nucleic acids from solution, and offer an alternative to synthesizing nanoparticles *de novo* in the laboratory as used in these prior studies.

The SPION capture method resulted in a lower DNA yield when compared to conventional phenol-based extraction and extraction with a commercial kit from 0.5 g soil samples. In contrast, Sebastianelli et al. (2008) and Paul et al. (2014) reported an increased yield, while Bandyopadhyay et al. (2011) reported a comparable yield to conventional and commercial extraction. The disproportional DNA yield between

methods in this study may be a result of the larger nanoparticle size (20 to 30 nm), which have a reduced surface area available for nucleic acid binding compared to nanoparticles of smaller diameters. There was no functional difference between quality measurements between the three groups, and the SPION capture method produced stronger PCR bands than the conventional phenol method, indicating this method is suitable for downstream molecular applications. Further experiments showed that 10 mg of Fe_3O_4 nanoparticles were the optimum quantity for use within the SPION capture method resulting in maximized DNA yield and minimized co-isolation of contaminants.

PVPP suspended in TE buffer and packed into a spin column was successfully removed PCR inhibitory substances in this study. Crude DNA was highly pigmented in all samples assessed before PVPP clean-up and did not produce PCR amplification (data not shown), while DNA in spin column elution was clear and of high quality. Therefore, we advocate the use of PVPP spin columns as beneficial in the development of a high throughput, effective method for purification of DNA from soil.

The SPION capture method detected *Meloidogyne* spp. DNA in samples inoculated with 100 and 1,000 nematodes in 90% of each replicate. The single sample in each of the 100 and 1,000 nematode densities that did not amplify contained residual pigments not fully removed by the PVPP spin column. This experiment identified a detection limit of 100 nematodes per 100 g of soil with high consistency. Moreover, the method was also capable of detecting *Meloidogyne* spp. DNA in samples inoculated with 1 and 10 nematodes in 20% and 40% of samples, respectively. These results suggest the method to be highly sensitive but detection is inconsistent in the presence of low nematode populations. The detection threshold may also be lowered if the downstream application has a higher sensitivity, such as quantitative PCR (qPCR), droplet digital PCR, or loop mediated isothermal amplification. Ophel-Keller et al. (2008) reported a detection limit of 2 nematodes per 100 g soil for *Ditylenchus dipsaci* using qPCR. Nonetheless, the

detection threshold of the 100g-SPION capture method developed here has utility for the model target organism, *M. hapla*, in the field. For example, damage thresholds reported for *M. hapla* in highly sensitive crops per 100 g soil include 15 to 190 second-stage juveniles for carrot (Gugino et al. 2006), 66 second-stage juveniles for potato (Olthof and Potter 1972), and 100 eggs for lettuce (Viaene and Abawi 1996).

Further refinements of this SPION capture method would be required for use by a muck soil type. In muck soil samples, the volume occupied by 100 g is approximately three times greater than an equal weight of mineral soil, resulting in greater absorption of the lysis buffer, and inability to collect lysis buffer by centrifugation. However, repeating the extraction with two and three times the amount of lysis buffer did not yield sufficient supernatant from the centrifugation step to result in DNA (data not shown). Modifications to determine the optimal ratio of soil to lysis buffer in the SPION capture method would be necessary for muck soils.

Additional advantages of the SPION capture method developed here are savings in time and cost. For example, the SPION capture method was 4 h faster than the other methods assessed and completed within 2 h. The estimated cost of performing one extraction from a 100 g soil sample using the SPION capture method was approximately $0.75 USD which is approximately 98% lower than the standard phenol method. The SPION method is also less technically challenging and complex than the standard phenol method and able to be completed on the laboratory bench and without necessitating use of a chemical fume hood.

In conclusion, the method described here using enzymatic laundry powder lysis, SPION capture, and PVPP purification offers a way to extract DNA from large volumes of soil with benefits of reduced heterogeneity between samples, and savings in time and cost without the need for hazardous chemicals compared to conventional extraction methods. Although the SPION capture method results in significantly lower quantities

of DNA compared to extraction using a standard phenol method or commercial kit for 0.5 g of soil, it was adapted for extraction of high quality DNA from 100 g soil with a detection limit of 100 nematodes per 100 g of soil. This limit is biologically relevant to growers making decisions regarding nematode control. The utility of this method is for isolation of high quality nematode DNA from large (100 g) soil samples, reducing variance and increasing accuracy of estimations due to spatial heterogeneity of the pathogen in the field facilitating molecular diagnostic detection and quantification of soilborne diseases.

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CHAPTER 3

DEVELOPMENT OF A SPECIES-SPECIFIC PCR FOR DETECTION AND QUANTIFICATION OF *MELOIDOGYNE HAPLA* IN SOIL USING THE *16D10* ROOT-KNOT NEMATODE EFFECTOR GENE*

Abstract

The Northern root-knot nematode (*Meloidogyne hapla*) is an important soilborne pathogen of numerous agricultural crops in temperate regions. Accurate detection and quantification is vital to supporting informed pest management decisions. However, traditional methods of manual nematode extraction and morphology-based identification are time consuming and require highly specialized training. Molecular methods may expand the diagnostician's toolkit beyond those methods that rely on this disappearing specialized skillset. However, molecular assays targeting the internal transcribed spacer region may lead to inaccurate results due to intraspecific variability. The *Meloidogyne* spp. effector gene *16D10* was assessed as a target for a SYBR Green-I quantitative PCR (qPCR) assay for detection and quantification of *M. hapla*. *M. hapla*-specific qPCR primers were developed and evaluated for specificity against five *M. hapla* isolates and 15 other plant-parasitic nematodes. A standard curve was generated by relating quantification cycle to the log of *M. hapla* population densities artificially introduced into soil. The influence of soil inhibitors on quantitative amplification was assessed by generating a dilution series from DNA extracted from pure nematode cultures and inoculated soil. Extracts from soil produced significantly higher Cq values than those produced from pure culture extracts. The utility of the qPCR was evaluated using soil samples collected from three naturally infested potato fields, resulting in a significant positive relationship between populations estimated using qPCR and

populations derived from manual counting. The qPCR developed in this study provides a useful method for detecting and quantifying *M. hapla* in soil, and demonstrates the utility of effector genes in plant-parasitic nematode diagnostics. The ability to use effector genes as targets for qPCR and other molecular detection and quantification methods may open additional avenues of novel research and support development of improved species level diagnostics.

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Introduction

Root-knot nematodes (RKN; *Meloidogyne* spp.) are sedentary endoparasites in which second stage juveniles (J2s) invade roots, establish specialized feeding sites, and pass through two further juvenile stages (J3 and J4) to become adults. Adult females are swollen and release eggs in a gelatinous matrix outside the root surface (Perry et al. 2009; Jones et al. 2013). Second-stage juveniles and males constitute the motile life stages. Typical symptoms of RKN include galls on roots and reduced root volume resulting in nondescript aboveground symptoms such as chlorosis, wilting, and stunting (Duncan and Phillips 2009).

The Northern root-knot nematode, *Meloidogyne hapla* Chitwood, has a broad host range and cosmopolitan occurrence, yet is of particular importance in temperate agricultural regions (Perry et al. 2009). *M. hapla* can cause significant reductions in yield and crop quality on a broad range of economically important annual crops including *Solanum* spp. (nightshade family, including potato (*S. tuberosum*); Van der Beek et al. 1998), carrot (Vrain 1982; Sapkota et al. 2016), and soybean (Barker and Olthof 1976), and numerous perennial crops, including grapevine (Walker 1997) and coffee (Handoo et al. 2005). Reductions in yield vary with crop and production region. In tomato, Barker et al. (1976) measured yield losses of up to 50% in microplots infested with 25 eggs/cm^3 of soil. In carrot and lettuce, two highly sensitive crops, damage and yield reductions exceeded economic thresholds at < 10 eggs/cm^3 (Viaene and Abawi 1996; Gugino et al. 2006). Many weed species and some cover crops, such as alfalfa, are also host to *M. hapla* (Griffin and Elgin 1977; Rich et al. 2008), making the development of comprehensive management strategies complex.

Effective management of *M. hapla* primarily relies on strategies prior to planting. A commonly used management strategy consists of soil treatment, with either a fumigant (e.g., methyl bromide, metam sodium) or non-fumigant (e.g., oxamyl) nematicides

(Mitkowski and Abawi 2003). Management strategies may also include rotation to a non-host crop such as cereals (Van der Beek et al. 1998), or field fallow, which may attenuate populations by removing the presence of a susceptible host. The primary objective of these strategies is to reduce initial *M. hapla* populations below the preplant economic threshold to limit the damage in the following crop (Barker and Olthof 1976; Madden et al. 2007). In many cropping systems, a significant inverse correlation describes nematode population densities prior to planting and crop loss (Seinhorst 1965; Barker and Olthof 1976). High population densities prior to, or at, planting may lead to an increased infection rate during initial stages of the epidemic, leading to high disease severity and damage (Madden et al. 2007). This relationship has been observed for *M. hapla* in many pathosystems including potato, tomato, and carrot (Olthof and Potter 1972; Barker et al. 1976; Wheeler et al. 1994; Gugino et al. 2006). Therefore, accurate pathogen detection and quantification is important for supporting informed disease management decisions and crop loss prediction models for *M. hapla* and other plant-parasitic nematodes. Within season control may include the use of resistant varieties and cultivars, yet few highly sensitive crops have significant practical resistance (Roberts 1992). For example, no genetic resistance to *M. hapla* is available in commercially cultivated potato (Janssen et al. 1995; Van der Beek et al. 1998; Melakeberhan et al. 2007).

In developing an integrated management plan for *M. hapla*, accurate and rapid detection and quantification of populations to the species level is critical (Madden et al. 2007). However, identification of plant-parasitic nematodes to species by observation of morphological characteristics is technically challenging and requires advanced training and experience (Min et al. 2012; Sapkota et al. 2016). Furthermore, quantification of populations using methods such as Baermann funnels or bioassays is time consuming (Ophel-Keller et al. 2008), requiring days to weeks to provide

information on nematode populations. Molecular identification of species provides an attractive alternative to morphology-based methods. Detection and differentiation of economically important *Meloidogyne* spp. have been accomplished using molecular-based techniques, such as restriction fragment analysis (Curran et al. 1986), polymerase chain reaction (PCR; Zijlstra 1997; Dong et al. 2001; Wishart et al. 2002), quantitative PCR (qPCR) (Hay et al. 2016; Sapkota et al. 2016; Sawada et al. 2011), loop-mediated isothermal amplification (LAMP; Niu et al. 2011; Peng et al. 2017), random amplified polymorphic DNA fingerprinting (RAPD; Randig et al. 2001; Adam et al. 2007), and high-resolution melt curve analysis (Holterman et al. 2012). Molecular methods offer advantages in being able to process many samples in parallel with a reduced processing lag time, and circumvent diagnostic limitations of RKN species based on morphology at the juvenile stage, which may lack distinctive features necessary for diagnostic certainty. Quantitative PCR offers additional advantages in simultaneous detection and quantification of DNA from a range of media to enable estimation of populations.

Numerous qPCR assays have been described for the detection and quantification of important plant-parasitic nematodes, including *Globodera rostochiensis* (Toyota et al. 2008; Goto et al. 2010), *M. javanica* (Berry et al. 2008), *M. incognita* (Sawada et al. 2011), *M. fallax* (Zijlstra and Van Hoof 2006; Hay et al. 2016), *M. hapla* (Hay et al. 2016; Sapkota et al. 2016), *M. enterolobii* (Braun-Kiewnick et al. 2016), *M. chitwoodi* (Zijlstra and Van Hoof 2006), *Paratrichodorus allius* (Huang et al. 2017), *Pratylenchus zeae* (Berry et al. 2008), *P. thornei* (Yan et al. 2010), *P. penetrans* (Mokrini et al. 2013; Baidoo et al. 2017), *P. neglectus* (Yan et al. 2013), *P. scribneri* (Huang and Yan 2017), *Ditylenchus destructor*, *D. dipsaci*, and *D. gigas* (Jeszke et al. 2015), and *Xiphinema elongatum* (Berry et al. 2008). These qPCR assays use the internal transcribed spacer (ITS) ribosomal DNA (rDNA) region as a target for primer and probe sequences. Intraspecies variability of the ITS rDNA region has been observed in many plant-

parasitic nematode species, including *Nacobbus aberrans* (Anthoine and Mugniéry 2005), *Bursaphelenchus* spp. (Prospero et al. 2015), *P. neglectus* (Hafez et al. 1999), and *Ditylenchus* spp. (Huang et al. 2010; Jeszke et al. 2015). Mandani et al. (2010) noted that populations of *Globodera rostochiensis* contained several ITS haplotypes, concluding the ITS region is not an ideal target region. Other studies have also reported a high degree of heterogeneity within the ITS rDNA region of other genera, even within individual nematodes (Zijlstra et al. 1995; Powers et al. 1997; Hugall et al. 1999). Regarding RKN, Kiewnick et al. (2014; 2015) and Holterman et al. (2012) suggested that due to the intraspecies variability measured in the ITS, this region is suitable for identification at the genus level, but not the species level. Conversely, some research has noted that *Meloidogyne* spp. are highly similar within the ITS. Handoo et al. (2005) were unable to distinguish *M. javanica* from *M. hapla* within the ITS region due to little polymorphism between species. Moreover, García and Sánchez-Puerta (2012) were unable to distinguish between *M. arenaria*, *M. javanica*, and *M. incognita*. Identification and quantification of *Meloidogyne* spp. and other plant-parasitic nematodes based on the ITS rDNA therefore has limited utility as a result of error from intraspecies variability and heterogeneity, potential non-specific amplification among non-target nematode species, and potential underestimation of true population densities. For example, the qPCR developed for *M. hapla* based on the ITS region by Sapkota et al. (2016) reported low specificity and a cross-reaction with *M. minor*. Moreover, the qPCR developed for *M. hapla* detection by Hay et al. (2016) did not observe cross reactions with similar species *in silico*, yet the species specificity of the assay *in vitro* using nematode isolates was not investigated.

Other molecular markers in taxonomically informative regions have been successfully used for species-level discrimination, with each offering unique advantages and limitations. Sequence characterized amplified regions (SCAR) have been used in

conventional PCR and qPCR to distinguish *Meloidogyne* spp. (Zijlstra 2000; Agudelo et al. 2011), yet these are not suited to multiplexing or high-throughput analysis (Braun-Kiewnick and Kiewnick 2018) and in some cases may be difficult to amend to a quantifiable assay such as qPCR. The cytochrome oxidase I and II (COI and COII) genes in the mitochondrial DNA are conserved and can provide a species-specific assay (Kiewnick et al. 2015; Toumi et al. 2015). Additionally, the large subunit (D2-D3) and small subunit (D1-D2) ribosomal DNA are highly conserved and are important targets for designing species-specific molecular assays (Rybarczyk-Mydlowska et al. 2012; Kiewnick et al. 2014; 2015). Similar to the ITS region, the intergenic spacer regions (IGS1 and IGS2) have been used to identify *Meloidogyne* spp. (e.g., Petersen et al. 1997; Wishart et al. 2002), yet the regions have also shown intraspecies variability and may not be suitable for species-level discrimination (Braun-Kiewnick and Kiewnick 2018).

Many economically important plant-parasitic nematodes species are obligate biotrophs that initiate close parasitic relationships with their hosts (Jones et al. 2013; Mitchum et al. 2013). During root penetration, migration, and feeding site establishment, plant-parasitic nematodes secrete numerous types of effector molecules (Mitchum et al. 2013). These effector molecules are comprised of small proteins or molecules that alter the host's cell structure and metabolism to assist in the nematodes' entry into and feeding from plant cells (Mitchum et al. 2013; Rehman et al. 2016), resulting in cell damage and reduced root function. Effectors involved in host modification include chorismate mutase (Lambert et al. 1999; Yu et al. 2011), endoglucanases (Gao et al. 2004), *16D10* in *Meloidogyne* spp. (Huang et al. 2006a), and pectate lyases (Bakhetia et al. 2007; Vanholme et al. 2007). Other secreted effector molecules suppress host immune responses or inactivate host-produced nematicidal compounds, allowing the nematode to feed with impunity (Smant and Jones 2011; Haegeman et al. 2012). Although some effector genes have homologs in bacteria and

fungi derived from early horizontal gene transfer events, many others are specific to plant-parasitic nematodes (Haegeman et al. 2012). Furthermore, unique species of plant-parasitic nematodes produce distinctive effectors (Smant and Jones 2011), which may be harnessed for molecular diagnostics. For example, Yu et al. (2011) were successful in distinguishing between the cyst nematode species *Globodera rostochiensis*, *G. pallida*, and *G. tabacum* using the chorismate mutase effector gene found in cyst and RKN species (Jones et al. 2003). Further, Mokrini et al. (2013; 2014) were able to design a specific and sensitive assay for detection of the root-lesion nematodes *P. penetrans* and *P. thornei* using the β-1,4-endoglucanase gene. Here, we investigate the use of the *Meloidogyne* spp. effector gene *16D10* as a target for the detection and quantification of *M. hapla* in qPCR to explore the potential of a broader range of molecular markers for plant-parasitic nematode diagnostics. The *16D10* gene encodes a secreted peptide thought to interact with a plant transcription factor to induce host root growth (Huang et al. 2006a; 2006b) and shown to be essential for parasitism in *Meloidogyne* spp. (Huang et al. 2006b).

The primary objective of this study was to develop and validate a qPCR for the species-specific detection of *M. hapla* using the *16D10* effector gene. A secondary objective was to evaluate the potential of this assay to detect and quantify *M. hapla* from DNA extractions conducted from soil.

Materials and Methods

Preparation of nematodes and DNA isolation from pure cultures and soil. To isolate DNA from pure cultures, *M. hapla* was reared and maintained in a hydroponics system (Gorny et al. 2018; Appendix A). *M. hapla* J2s were observed under a microscope, removed from the reservoir water with an ultra-fine dental pick (Dentsply Flexofile size 006, 25 mm), and placed in 500 µl sterile Type I ultrapure water. The

dental pick was examined after each transfer to ensure nematode deposition. The nematode suspension was centrifuged at 10,000 *g* for 5 min and the supernatant removed. The remaining moisture was removed by centrifuging for 15 min in a vacufuge concentrator (Eppendorf, Hauppauge, NY). DNA isolation from the nematode pellet was performed as described by Yan et al. (2008) with slight modifications described here. Briefly, in a 1.5 ml microcentrifuge tube, 80 µl of lysis buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM MgCl2, 10 mM dithiothreitol, 4.5% Tween-20, and 0.1% gelatin) was added and the mixture vortexed briefly to disrupt the pellet. The lysis buffer suspension was incubated at -20°C until frozen. Suspensions were then thawed and 20 µl of Proteinase K (20 mg/ml) was added. The mixture was then incubated at 65°C for 1 h, transferred to 95°C for 10 minutes to deactivate the Proteinase K, centrifuged at 13,200 *g* for 5 min, and the supernatant containing the DNA template transferred to a new tube.

To isolate DNA from soil, *M. hapla* J2s for each experiment were similarly inoculated onto 0.5 g of autoclaved soil mix (three parts top-soil to one part sand; Sensenig Landscape Supply, Geneva, NY). Genomic DNA was isolated from the inoculated soil using the DNeasy PowerSoil DNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, with the modification that 0.5 g soil was used and the final elution volume was 60 µl.

DNA concentrations were determined using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). Resultant DNA from soil and pure culture, and DNA of other *M. hapla* isolates and non-target nematode species (Table 3.1), was stored at -20°C in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.3).

Development of species-specific primers. All available sequences of the *16D10* effector gene from *Meloidogyne* spp. were obtained from GenBank (*M. javanica*, DQ841121; *M. arenaria*, DQ841122; *M. hapla*, DQ841123 (Huang et al. 2006b); and

M. incognita, DQ087264 (Huang et al. 2006a) and aligned using ClustalW within the software Geneious™ v. 8.1.2 (Kearse et al. 2012). Regions of polymorphism between *M. hapla* and the other *Meloidogyne* spp. were assessed for placement of primer binding locations for conventional and qPCR. Primer pairs (forward and reverse) were designed to unique regions within the *16D10* sequence among the different *Meloidogyne* species using the software Primer3 (v. 4.1.0; Koressaar and Remm 2007; Untergasser et al. 2012). Primer sets were evaluated for annealing temperatures and guanine-cytosine (GC) content, and sets were assessed for self-priming and dimer formation using OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA). Primer pairs were screened for potential mis-priming and non-target priming using the Primer BLAST function of GenBank. One set, designated as Mha17, was identified as having potentially optimal reaction conditions, a high degree of *in silico* species-specificity, and low degree of self-polymerization. The primer set Mha17 consisted of the forward Mha17f (5'-tgaatagttggtggcctctg-3') and reverse Mha17r (5'-tgtgctatttccaagggtaaag-3'). Primers were synthesized by Integrated DNA Technologies.

Primer specificity. The specificity of the Mha17 primers was assessed against DNA from five *M. hapla* isolates and 15 non-target plant-parasitic nematode species using conventional PCR from single nematode DNA suspensions (Table 3.1). Non-target plant-parasitic nematodes were selected on the basis of their ubiquitous prevalence in potato field soils. Reaction mixtures (15 µl) contained 1× Standard *Taq* Reaction Buffer (New England Biolabs), 5 mM dNTPs, 1 U *Taq* DNA polymerase (New England Biolabs), 3 mM each of the Mha17 forward and reverse primers, and 2 µl template DNA. The reaction cycle conditions were an initial denaturation step of 95°C for 3 min, followed by annealing and extension over 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The optimal annealing temperature was determined prior to this reaction cycle through a gradient

PCR (53 to 63°C). Amplification of the correct target region was first confirmed through separation of PCR products on a 1.5% agarose gel stained with 1× GelRed at 50 V for 120 min. The gel was visualized under ultra-violet light and the band size was compared to a 100 bp ladder (Axygen Scientific, Corning Inc., Corning, NY). Quality of the nematode DNA was checked by performing conventional PCR using the ITS primers rDNA2 and rDNA1.58s (Powers et al. 1997) using the same reaction mixture as above. The reaction cycle conditions for amplification of the ITS region consisted of an initial denaturation step of 95°C for 5 min, then 34 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 5 min.

To confirm amplification of the target region, PCR products from *M. hapla* generated using the Mha17 forward and reverse primers were cloned into a pMiniT vector maintained in *E. coli* using a PCR Cloning Kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Colonies (n = 35) were screened for successful insert of the PCR product and five colonies were selected for plasmid sequencing. Plasmid DNA was extracted using an E.Z.N.A. Plasmid Mini Kit I (OMEGA Bio-Tek, Norcross, GA) and sequenced using the cloning analysis primers included with the PCR Cloning Kit. Sequencing of amplicons (in both directions) was performed at the Cornell University Biotechnology Resource Center (Ithaca, NY) on an Applied Biosystems 3730xl DNA analyzer. Consensus sequences were assembled within Geneious and aligned with full gene sequences of *M. hapla 16D10* (full gene sequence, 609 bp) using the BLAST search function of GenBank.

Table 3.1. Meta-data associated with the plant-parasitic nematodes selected to test specificity of the quantitative PCR (qPCR) primers (Mha17f/Mha17r) developed for detection and quantification of *Meloidogyne hapla*.

Species	Origin	Source	+/- PCR Amplification[a]	+/- qPCR Signal[b]
Meloidogyne hapla	New York	S. Pethybridge	+	+ (34.39)
M. hapla	New York	S. Pethybridge	+	+ (36.26)
M. hapla	New York	S. Pethybridge	+	+ (35.74)
M. hapla	Netherlands	A. Skantar	+	+ (28.54)
M. hapla	Australia	N. Perry	+	+ (22.98)
M. arenaria	Delaware	A. Skantar	-	-
M. arenaria	New York	L. Cadle-Davidson	-	-
M. chitwoodi	Washington	A. Skantar	-	-
M. incognita	Dominican Republic	A. Skantar	-	-
M. minor	Unknown	A. Skantar	-	-
M. naasi	California	A. Skantar	-	-
M. fallax	Belgium	A. Skantar	-	-
M. javanica	Unknown	N. Perry	-	-
M. enterolobii	North Carolina	E. Davis	-	-
Pratylenchus penetrans	New York	S. Pethybridge	-	-
P. neglectus	Unknown	A. Skantar	-	-
P. thornei	Oregon	A. Skantar	-	-
Heterodera schachtii	Oregon	A. Skantar	-	-
H. trifolii	Greece	A. Skantar	-	-
H. glycines	Delaware	A. Skantar	-	-
H. glycines	China	A. Skantar	-	-
Ditylenchus destructor	Unknown	A. Skantar	-	-

[a] Isolates marked as positive produced a single product at 110 bp on a electrophoresis gel after conventional PCR. Isolates designated as negative failed to produce a visible amplicon.

[b] Isolates marked as positive produced a positive signal during quantitative PCR. Isolates marked as negative produced no signal. Corresponding Cq values from each isolate producing a positive signal are included in parentheses.

Quantitative PCR. qPCR was performed on a Bio-Rad C1000 CFX96 (Bio-Rad Laboratories, Hercules, CA). Reaction mixtures consisted of 1× SsoAdvanced Universal Probe Supermix (Bio-Rad), 0.25 M betaine, 0.3 mM additional $MgCl_2$, 0.075× SYBR Green-I (Fisher Scientific, Pittsburgh, PA), 0.25 µM of each primer, 5.0 µl template DNA, and water to bring the reaction volume to 20 µl. Optimal concentration of the primers were determined in preliminary experiments (data not shown). Amplification conditions consisted of incubation at 95°C for 3 min, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s with fluorescence data collected following each combined annealing and extension step. The optimal combined annealing/extension temperature was determined through varying between 55 and 65°C; data not shown). A melting curve analysis was performed at the end of the cycling reactions (60 to 95°C, in 0.5°C increments). Positive (1,000 pg *M. hapla* DNA), negative (800 pg *M. arenaria* DNA), and no template negative (sterile Type I water) controls were included in each assay. A sample was considered negative if no rise in fluorescence was detected by cycle 45 of each run. Three replicates of each sample were included. Intra-assay variability was assessed by including five replicates of the same sample within one assay. Interassay variability was assessed by including the same sample across five separate reaction runs. Reaction conditions were controlled, quantification cycles (Cq) recorded, and specificity assessed using CFX Manager software program (Bio-Rad, version 3.1). Identity of the amplicon was also confirmed by separating products on a 1.5% agarose gel stained with 1× GelRed at 50 V for 120 min.

Generation and validation of population standard curve. To correlate the Cq value to *M. hapla* population densities within the soil, 1, 5, 10, 50, 100, and 500 *M. hapla* J2s were inoculated into 0.5 g of autoclaved soil, air dried for 3 days to remove excess moisture, then DNA extracted as described above for soil. Each inoculation level was

replicated five times and a non-inoculated soil extraction control was included. The samples were assayed in triplicate using the qPCR described above. Resultant Cq values were plotted against the log of the number of inoculated *M. hapla* individuals, and amplification efficiency calculated. The standard curve was validated by inoculating additional aliquots of 0.5 g soil with 3, 10, 25, and 250 *M. hapla* J2s, and a non-inoculated control. The log of predicted population densities from resultant Cq values and standard curve were plotted against the log of inoculated population densities.

Assay sensitivity and influence of inhibitory compounds. To investigate the sensitivity of the Mha17 primers (defined here as the lowest number of *M. hapla* J2s per extraction that produce a visible band on an electrophoresis gel after amplification), DNA was isolated from soil inoculated with 1, 5, 10, and 20 *M. hapla* J2s as described above for soil. Each inoculation level was replicated five times. PCR reactions (15 µl) were prepared and amplification assessed as described above for primer specificity testing using conventional PCR. PCR reactions were performed in duplicate.

To investigate the sensitivity of the qPCR assay and determine the influence of soil inhibitors on reaction efficiency, standard curve assays from serial dilutions of *M. hapla* DNA isolated from both pure culture and soil were performed. DNA was isolated from a suspension of 1,000 *M. hapla* J2s as described above for pure cultures. Serial dilutions of the DNA were prepared (1:1, 1:10, 1:100, 1:1,000, and 1:10,000 representing approximately 790 pg/µl to 0.079 pg/µl *M. hapla* DNA) using sterile Type I water. DNA concentrations were determined using a Qubit fluorimeter. A similar dilution series was prepared using DNA extracted as described for soil from 1,000 *M. hapla* J2s inoculated onto 0.5 g sterilized soil. qPCR reactions were performed as described, for each serial dilution step, from pure culture and soil extracts. Reactions were performed in triplicate, and each run included positive (1,000 pg *M. hapla* DNA), negative (800 pg *M. arenaria*

DNA), and no template negative (sterile Type I water) controls. Standard curves were constructed by plotting the dilution fold change against Cq value.

Assessment of field samples. To assess the utility of the Mha17 primers and qPCR in relating population density estimates derived from the qPCR with those from morphology-based identification, three commercial potato fields in western New York State with previous histories of *M. hapla* infestations were selected. One week after planting to potato (cvs. Eva and Genesee) soil samples (approximately 1 kg) were collected from 100 locations on a regular grid (15.2 m × 15.2 m between points) within each field. *M. hapla* population densities were quantified through a modified Whitehead tray (Whitehead and Hemming 1965) for each location. Briefly, a mesh support was nested inside a solid base pastry baking pan. A milk filter (KenAG, Ashland, OH) and a single-ply facial tissue (Kleenex; Kimberly-Clark Corp., Irving, TX) were placed on the mesh support. A subsample of field soil (200 g) was placed on the supports, and distilled water (approximately 300 ml) was added to the lower pan, gently wetting the soil through the mesh, milk filter, and tissue. Soil was incubated in the dark for 48 h, after which nematodes were collected by passing the water twice through a 25 µm mesh sieve held at a 45° angle. Collected nematode suspensions were adjusted to 25 ml, and 5 ml removed for quantification under 400× magnification, and populations calculated per 200 g soil subsample. Ten locations with high populations and ten locations with low populations, as determined by manual extraction followed by morphology-based enumeration, were selected from across the three fields for assessment using the Mha17 primers and final qPCR conditions described above.

Statistical analysis. Statistical analyses were performed within R (version 3.3.3; R Core Team 2017) after logarithmic transformation of *M. hapla* population densities. The population standard curve was generated by regressing nematode densities and Cq values obtained from qPCR. The robustness of the standard curve was tested by

conducting linear regression analysis to describe the relationship between inoculated validation nematode densities and assay-predicted nematode densities. Differences between Cq values of serial dilutions from DNA extracts from pure culture and soil was explored using Student's *t*-test at each dilution. The relationship between *M. hapla* population densities predicted from morphology-based counting and qPCR was explored through linear regression.

Results

Development of species-specific primers. Alignment of sequences of the *16D10* gene from *Meloidogyne* spp. identified unique regions of polymorphism used to develop species-specific primers for detection and quantification of *M. hapla* in a qPCR assay. These primers amplified a 110 bp region of the *16D10* gene, outside of the predicted coding region. The GC content for the forward and reverse primers was 50% and 40.9%, respectively, and the estimated annealing temperatures were 54.5 and 52.8°C, respectively. The primers exhibited no matches to non-target plant-parasitic or free-living nematodes, and no strong similarity to other soilborne microorganisms using the BLAST search function of NCBI (Mha17f and Mha17r sequences E-values ≤ 0.3; next closest alignments E-values ≥ 1.2).

Primer specificity. The primer pair designated Mha17 produced a single amplification band in all *M. hapla* isolates tested. No amplification was observed in other *Meloidogyne* spp. or in other non-target plant-parasitic nematodes assayed (Table 3.1). Melt curve analysis identified a single peak at 78.5°C (Fig. 3.1). Sequencing of cloning products produced nucleotide sequences that were a perfect match to the *M. hapla 16D10* gene sequence DQ841123 in GenBank.

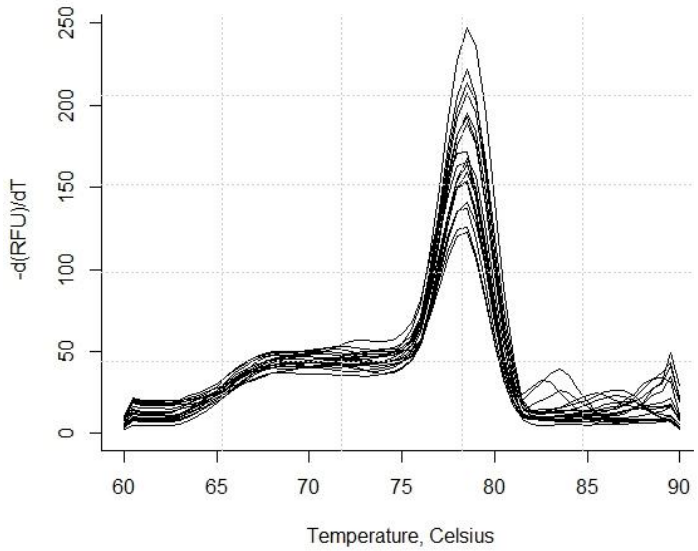

Fig. 3.1. Melting curve of *Meloidogyne hapla* amplicons with a melting temperature of 78.5°C using the Mha17f/Mha17r primers developed in this study and DNA of an isolate of *M. hapla* from New York State. Reactions not containing *M. hapla* DNA did not produce a signal.

Quantitative PCR. The coefficient of variation for intra-assay and interassay variability was 1.35% and 4.22%, respectively.

Generation and validation of population standard curve. A standard curve generated by plotting the Cq values derived from soil inoculated with 1, 5, 10, 50, and 500 *M. hapla* J2s per 0.5 g soil (y = -2.949x + 37.01, R^2 = 0.9, P = 0.009; Fig. 3.2). The efficiency of the reaction was 118.3% and Cq values ranged between 29.19 and 44.13. No amplification was observed in the non-inoculated soil or non-target species controls. The population standard curve was validated with DNA isolated from soil inoculated with 3, 10, 25, and 250 *M. hapla* J2s per 0.5 g soil. A significant linear relationship was observed between the log inoculated population density and the log estimated

population density derived from the standard curve ($y = 1.281x + 0.001$, $R^2 = 0.87$, $P = 0.0009$; Fig. 3.3). No amplification was observed in the non-inoculated or non-target species controls.

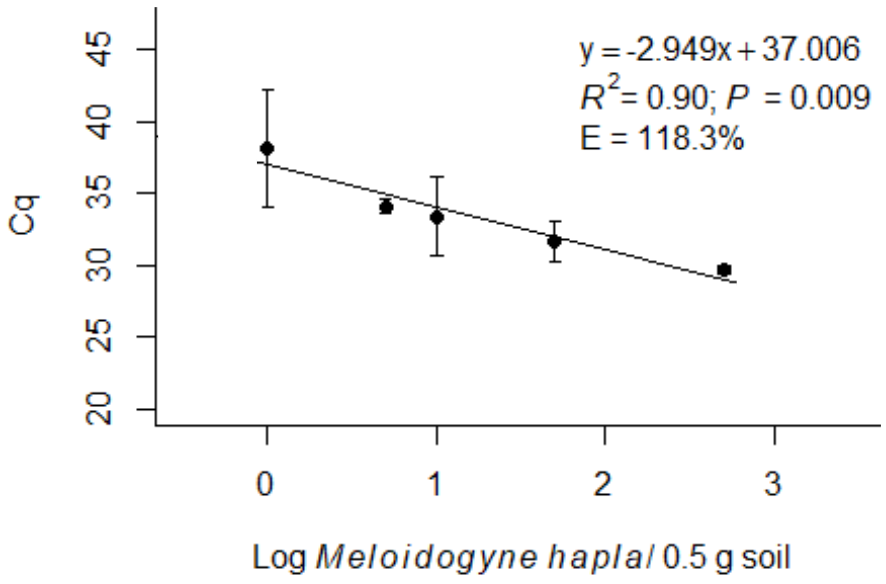

Fig. 3.2. A standard curve of the quantitative PCR (qPCR) using the Mha17f/Mha17r primers for detection and quantification of *Meloidogyne hapla* based on the *16D10* effector gene. The log of the number of *M. hapla* second-stage juveniles (J2s) inoculated in 0.5 g soil (1, 5, 10, 50 and 500 *M. hapla* J2s) is plotted against the resultant quantification cycle (Cq) value. Five replicates of each inoculation level were performed, and the qPCR was conducted in triplicate.

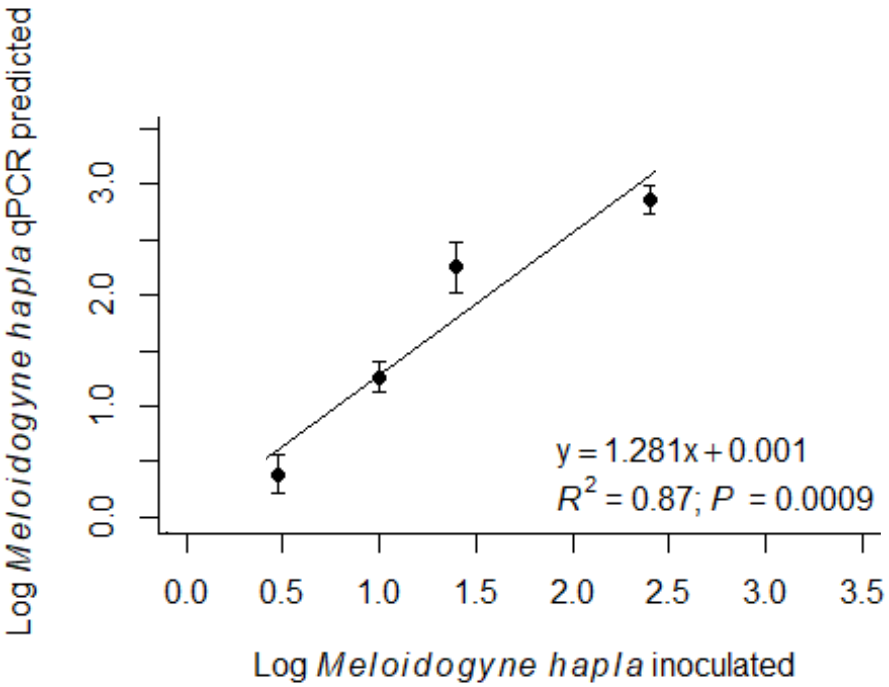

Fig. 3.3. The population standard curve based on the Mha17f/Mha17r primers targeting the *16D10* effector gene was tested by inoculation of *Meloidogyne hapla* second-stage juveniles (3, 10, 25 and 250) per 0.5 g soil and calculation of predicted population density from resultant Cq values.

Assay sensitivity and limit of detection. Amplification of the target region of *16D10* through conventional PCR occurred in three of five samples inoculated with one nematode, all samples inoculated with five nematodes, four of the five samples inoculated with 10 nematodes, and four of the five samples inoculated with 20 nematodes per 0.5 g soil. No amplification was observed in the non-inoculated control.

Standard curves were generated from serial dilutions of DNA isolated from pure cultures of 1,000 *M. hapla* J2s and soil inoculated with 1,000 *M. hapla* J2s, each

representing a range of DNA concentrations from approximately 790 pg to 0.079 pg/µl. The curve derived from assays of pure culture DNA extracts was described by: $y = -3.065x + 22.738$ ($R^2 = 0.99$, $P < 0.001$). The curve derived from assays of soil DNA extractions was described by: $y = -3.656x + 28.596$ ($R^2 = 0.68$, $P = 0.05$). Student's *t*-test analysis between mean Cq values resulted in a significant difference between soil and pure culture extracts at all dilutions except for the least dilute (1:1) level (Table 3.2), indicating that amplification inhibitors were present in a greater concentration within soil extracts, leading to higher Cq values.

Table 3.2. The effect of soil inhibitors on the quantitative PCR (qPCR) assay for the detection of *Meloidogyne hapla* using the Mha17f/Mha17r primers targeting the *16D10* effector gene was assessed. DNA was isolated from *M. hapla* second-stage juveniles (J2s) in pure culture and from *M. hapla* J2s inoculated to soil. Serial dilutions were made from isolation products from approximately 790 to 0.079 pg/µl *M. hapla* DNA.

Dilution	Cq Soil[a]	Cq Pure Culture[b]	*t*[c] (*P* value)
1:1	30.93	22.74	3.44 (0.17; ns)
1:10	31.16	25.59	5.40 (0.002)
1:100	35.06	28.99	4.13 (0.02)
1:1,000	35.20	31.28	3.25 (0.04)
1:10,000	47.19	35.48	8.43 (0.04)

[a] Mean Cq value for dilutions from soil isolations.
[b] Mean Cq values for dilutions from pure culture isolations.
[c] Student's *t*-value.

Assessment of field samples. Nematode population density determined by manual counting and qPCR displayed a significant positive relationship ($R^2 = 0.41$, $P < 0.001$), and was described by the equation: $y = 0.64x - 1.06$ (Fig. 3.4).

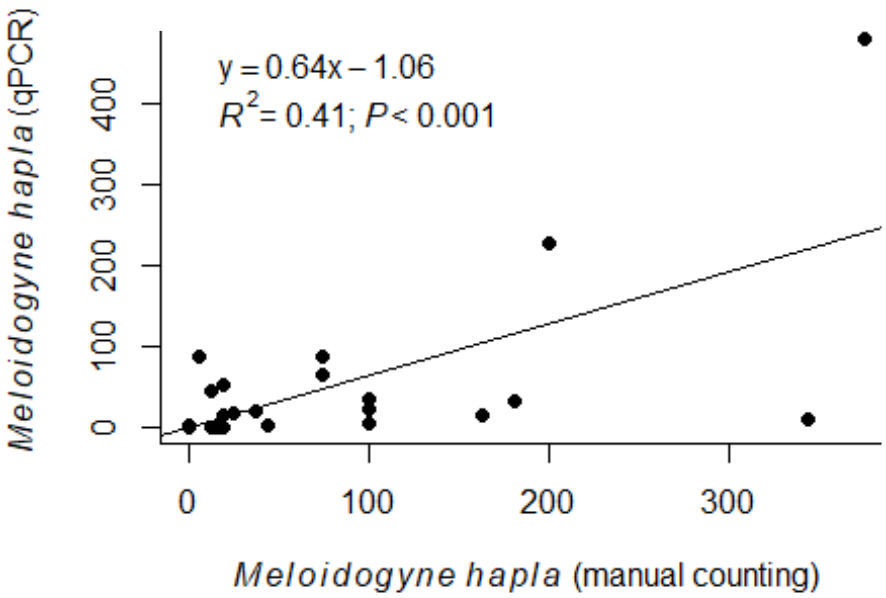

Fig. 3.4. Relationship between *Meloidogyne hapla* population density estimates derived from morphology-based identification and from the quantitative PCR assay to evaluate the utility of the Mha17f/Mha17r primers for detection of *M. hapla* from potato fields in New York State.

Discussion

Quantitative PCR remains an important tool in plant pathology research and diagnostics, circumventing potential problems related to accurate identification and quantification of plant-parasitic nematode populations. Yet, the selection of a target region for a qPCR assay should be guided by the goals and objectives of the application, such as specific management questions or broader taxonomic studies (Ahmed et al. 2016; Braun-Kiewnick and Kiewnick 2018). Here, an accurate and reliable SYBR Green I qPCR based on the *16D10* effector gene was developed for the detection and quantification of *M. hapla* from soil. The *16D10* gene has been reported as unique to

Meloidogyne spp. but with up to 30% sequence dissimilarity between orthologs in some species (Dinh et al. 2015). Sufficient polymorphism was observed between published sequences of the economically important species *M. hapla*, *M. javanica*, *M. arenaria*, and *M. incognita* to allow for the development of qPCR primers for detection of *M. hapla*. Testing with end-point and qPCR confirmed the primers were highly species-specific, exhibiting no cross reactivity with the other *Meloidogyne* spp. or plant-parasitic nematodes tested. The primers also amplified *M. hapla* isolates from diverse geographies, indicating the assay has potential for use in diagnostics across broad regions. This degree of specificity is an improvement over ITS-based assays in which cross-reactivity of the primers with non-target species was noted (e.g., Sapkota et al. 2016) and was of similar sensitivity to the ITS-based assay used by Hay et al. (2016). Indeed, similar improvements were observed by Yu et al. (2011) in a qPCR assay using the chorismate mutase effector gene for detection of selected *Globodera* species. However, in sensitivity experiments using conventional PCR, only four of five samples of those inoculated with either ten or 20 *M. hapla* J2s produced visible product bands on an electrophoresis gel. This was likely due to potential inhibition of the sample rather than lack of sensitivity of the primers. Although this study provides additional support to the hypothesis that effector genes within plant-parasitic nematodes may be utilized as highly specific targets for detection and quantification, perspective should be retained; the *16D10* gene is still a relatively new area of effector research, with few published sequences available. A more comprehensive phylogenetic analysis of the *16D10* gene among additional *Meloidogyne* species and regional isolates of *M. hapla* is ultimately needed to understand potential intraspecific gene variation within *16D10* and support a highly robust diagnostic test.

Huang et al. (2006a) predicted a gene copy number of *16D10* of three to four in *Meloidogyne* spp., based on DNA hybridization studies. The lower copy number of

16D10 compared to the ITS region may account for the generally higher Cq values observed in this study when compared to similar qPCR assays based on the *Meloidogyne* spp. ITS rDNA region, which is present in multiple copies (Blok 2005; Skantar and Carta 2005). A high degree of variability was observed between some replicates in the current study. The presence of *16D10* at low copy number may preclude stochastic partitioning of template molecules into the reaction wells, resulting in greater variability, particularly for samples with a low nematode population density.

The effect of soil on reaction efficiency was evaluated and compared to manual extraction and morphology-based identification and quantification of *M. hapla*. The assay was highly sensitive, and able to detect one *M. hapla* J2s in 0.5 g of soil as determined through end-point PCR, with a sensitivity of approximately 0.079 pg/µl. This sensitivity is comparable to ITS-based qPCR assays. For example, Sapkota et al. (2016) reported a lower limit of detection of 0.5 pg for detection of *M. hapla*. The sensitivity of the qPCR developed here can support the economic threshold range of many susceptible crops, including carrot and lettuce, which have estimated action thresholds less than 10 eggs/cm^3 (Viaene and Abawi 1996; Gugino et al. 2006). However, the variation in samples containing one *M. hapla* J2 per 0.5 g of soil was high, indicating that several replicates are required to ensure accurate estimates. Using end-point PCR, the Mha17 primers exhibited a consistent lower limit of detection of five nematodes in 0.5 g soil. Although the qPCR method described here was developed using J2s, many economic threshold values for populations of plant-parasitic nematodes are reported as eggs per volume of soil. Yan et al. (2013) found no significant difference in the Cq values produced from different life stages of the root-lesion nematode *P. neglectus*, suggesting that given a suitable DNA isolation method, different life stages may be quantified accurately. Yet this remains to be confirmed within *Meloidogyne* spp.

In this study, the Cq values from reactions using DNA isolated from pure culture were up to 11.7 cycles earlier than those using DNA isolated from soil using a commercial DNA extraction kit, suggesting that amplification inhibitors remained after soil DNA extraction. Further, the amplification efficiency for samples isolated from soil was 118.3%, above the expected 100%. This increase in amplification efficiency is thought due to the action of polymerase inhibitors (Svec et al. 2015), indicating a reduction in the ability of the DNA extraction method to sufficiently remove contaminants. Soil type may also influence extraction efficiency, as it is observed that soils with high humic acid content (such as those with high clay content) are more difficult to separate contaminants (e.g., Zhou et al. 1996; Miller et al. 1999). Delayed amplification reflected by higher Cq values and variability using DNA extracts from soil also has the potential to be improved by more efficient soil DNA extraction and purification protocols, such as the addition of polyvinylpolypyrrolidone to the extraction buffer (Steffan et al. 1988; Zhou et al. 1996). It is therefore recommended that when deriving estimates of *M. hapla* population densities from soil DNA extracts using the method described here, one should account for this higher degree of variability and suboptimal amplification efficiency by including additional soil biological replicates and qPCR technical replicates within the assay. Within this, determining the optimal sampling density within an agricultural field for estimation of *M. hapla* population densities using this qPCR assay while maintaining cost effectiveness is a logical next step.

A significant relationship was observed between *M. hapla* population estimates obtained by manual counting and qPCR, yet the sum of squares value was less than one, indicating deviation about the regression line. Indeed, some locations having high nematode counts in the manual extraction did not have corresponding high estimates within the qPCR assay. This variation may have resulted from inefficiencies and inconsistencies inherent to manual nematode extraction methods and potential

nematode misidentification (Yan et al. 2012; Hay et al. 2016). The potential for error is also introduced into estimated values due to the log-transformation of populations (Toyota et al. 2008). The qPCR may therefore be more reliable for quantifying fold changes in nematode population densities, but smaller changes may be inherently less accurate.

The qPCR for detecting and quantifying *M. hapla* presented here provides an alternative for the need for lengthy manual extractions and error-prone morphology-based identification, and is a resource for the continued monitoring of this species in many economically important agricultural crops. The use of effector genes as targets for qPCR provides species-specific quantification, and advances nematode detection technologies by expanding the range of novel qPCR target regions.

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CHAPTER 4

SPATIAL AND SPATIOTEMPORAL ANALYSIS OF *MELOIDOGYNE HAPLA* AND *PRATYLENCHUS* SPP. POPULATIONS IN COMMERCIAL POTATO FIELDS IN NEW YORK STATE*

Abstract

Meloidogyne hapla and *Pratylenchus* spp. are important plant-parasitic nematodes affecting potato in New York State and the Northeastern United States, yet little is known of their spatial patterns and spatiotemporal dynamics. Spatial patterns of *M. hapla* and *Pratylenchus* spp. were quantified through geostatistical methods of semivariogram analysis and ordinary kriging, and Spatial Analysis by Distance IndicEs (SADIE) within each of three commercial potato fields on two occasions, in 2016 and 2017. Semivariogram analysis and ordinary kriging indicated initial population densities to be spatially dependent over an average range of 110 m for *M. hapla* and 147 m for *Pratylenchus* spp. SADIE indicated populations of *Pratylenchus* spp. to be significantly aggregated in nearly all fields (10 of 12 samplings, I_a = 1.367 to 2.113). *M. hapla* populations were aggregated at only a few instances (3 of 12 samplings, I_a = 1.318 to 1.738). Spatiotemporal analysis using the Association Function of SADIE indicated a strong and significant association between initial and final population densities of *M. hapla* and *Pratylenchus* spp. within fields. This information lays the foundation for the development of enhanced sampling protocols for estimation of plant-parasitic nematodes in New York State potato fields, and aids in the cost-benefit analysis to evaluate the potential of site-specific nematicide application.

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Introduction

The Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.), are soilborne pathogens of potato and other vegetable crops that can cause extensive crop loss and damage in New York State and the Northeastern United States (Mitkowski et al. 2002; Bird et al. 2018). In potato, nematode feeding on plant roots may impact returns by reducing tuber weight or number (Dickerson et al. 1964; Olthof and Potter 1972; MacGuidwin and Rouse 1990; Philis 1995; Holgado et al. 2009). Nematodes may also deleteriously affect quality by inflicting cosmetic damage to tuber flesh or internal defects, rendering fresh market products unacceptable and lessening the quality of processing products (Ingham et al. 2007; King and Taberna 2013). Infection by *M. hapla* may lead to galls on roots (Perry et al. 2009), or in bumps and swelling of the tuber, predominantly in the surface tissue layers (Griffin and Jorgenson 1969). Infection by *Pratylenchus* spp. (particularly *P. penetrans*) may lead to necrotic lesions and root dieback (Bridge and Starr 2007; Jones et al. 2013) resulting in reduced root volume, and blisters and potato scab-like symptoms on tubers (Holgado et al. 2009).

In spatial ecology, organisms may assume patterns as a function of their biology and ecology, such as aggregated, uniform and random (Illian et al. 2008). Populations of plant-parasitic nematodes are typically aggregated (Goodell and Ferris 1980; Campbell and Noe 1985; Wallace and Hawkins 1994; Robertson and Freckman 1995), but may be distributed by movement of soil, water, or machinery, which can substantially alter spatial patterns over time. For example, Farias et al. (2002) observed reniform nematode (*Rotylenchulus reniformis*) populations to be aggregated within Brazilian cotton fields, with the degree of aggregation increasing between the initial and final sampling times. Webster and Boag (1992) found potato cyst (*Globodera rostochiensis*) and cereal cyst (*Heterodera avenue*) nematodes to be highly aggregated. Wallace and Hawkins (1994)

found several plant-parasitic nematode populations to be highly aggregated, including *P. penetrans*, *Tylenchorhynchus* spp., and *H. trifolii*, but found *Tylenchus maius* populations were random at the scale considered in the study (10 m).

Due to the high degree of spatial variability and the substantial investment of time and resources required to collect and analyze soil samples for nematode population densities and patterns, assumptions are frequently made on spatial distributions, lags, and population density parameters (i.e. mean and variance). This uncertainty may substantially affect the accuracy and precision of the estimates and ultimately management decisions (Wheeler et al. 1994; Robertson and Freckman 1995). An understanding of the spatial distribution and population density parameters of a pathogen is required for a comprehensive understanding of ecological and epidemiological processes and provide a basis for the development of appropriate sampling techniques and intensity (Steinberg and Kareiva 1997; Winder et al. 2001). For example, knowledge of the spatiotemporal characteristics of soilborne disease epidemics is useful for inferring pathogen introduction events and epidemic progress, including changes in population density and patterns as a function of field management (Goodell and Ferris 1980; Contina et al. 2018), and can add to the understanding of host-pathogen interaction and crop loss (Campbell and Noe 1985). Analysis of spatial patterns may also test hypotheses about associations among pathogen taxa such as co-occurrence or affinity (Pethybridge and Turechek 2003), environmental biology, or effects of abiotic factors such as soil moisture and texture on spatial patterns and crop loss (Campbell and Noe 1985). Knowledge of spatial patterns and scale may improve crop loss models and form the basis for determining the number of samples to accurately estimate mean pathogen population densities, and optimize sampling patterns, thus developing effective and economical monitoring strategies (Campbell and Noe 1985; Wheeler et al. 1994). Wheeler et al. (1994) collected data on populations of *M. hapla*

and *P. penetrans* from commercial potato fields and found *M. hapla* populations were best described by the negative binomial distribution, indicating aggregation. However, two of 10 *P. penetrans* populations were best described by the Poisson distribution providing support to a null hypothesis of a random spatial distribution (Campbell and Noe 1985; Wheeler et al. 1994). Wheeler et al. (1994) used this spatial information as a basis to suggest six to eight samples per 2 m × 2 row plot were needed to estimate the population to within 50% of the coefficient of variation of the mean, for both species.

In New York State and the Northeastern United States, management of plant-parasitic nematodes in potato is often through fumigant and non-fumigant nematicides (Mitkowski and Abawi 2003). Decisions regarding the use of these nematicides should be guided by the risk of disease and crop loss, due to their high cost and potentially harmful off-target effects, thus minimizing the rate of false positive applications. The risk of disease and crop loss have been positively correlated with nematode populations present early in the season (i.e. initial population densities; Seinhorst 1965; Oostenbrink 1966; Olthof and Potter 1972; 1973). A comprehensive knowledge of nematode population densities, spatial patterns, and spatiotemporal changes within the field is therefore important for evaluating management options. Spatially explicit information on plant-parasitic nematode populations may also support the development of targeted, site-specific nematicide application, reducing overall farm inputs and minimizing environmental impact (Farias et al. 2002). Several studies have assessed the utility of site-specific nematicide application, supported by detailed spatial distribution maps and associations between nematode population densities, crop loss, and edaphic factors (Evans et al. 2002; Avendano et al. 2003; Kulkarni et al. 2008; King and Taberna 2013). For example, King and Taberna (2013) reported a 30% reduction in nematicide usage by site-specific application for control of Columbia root-knot nematode (*M. chitwoodi*) in Idaho.

With logistical and economic constraints making it impossible to sample every location within a field, an alternative method for quantifying regionalized variables of interest within that space is needed. Statistical approaches for quantifying the spatial arrangement of continuous nematode population data include fitting frequency distributions (Campbell and Noe 1985; Madden et al. 2007), mapping and quantifying spatial autocorrelation (Campbell and Noe 1985; Cressie 1988; 1989; 1993; Contina et al. 2018), and calculating statistics to quantify aggregation and other patterns (e.g., Spatial Analysis by Distance Indices; Perry 1995; 1998).

The objective of this study was to quantify the spatial and spatiotemporal patterns of *M. hapla* and *Pratylenchus* spp. populations in commercial potato fields in New York State. This information is essential as a foundation for the design of appropriate sampling strategies to adequately depict populations for pre-plant risk assessments and planning cropping rotation, and evaluate the potential for site-specific application of nematicides for economic and environmental sustainability.

Materials and Methods

Field study sites and data collection. Three commercial potato fields were intensively sampled in each of two years, 2016 and 2017, for *M. hapla* and *Pratylenchus* spp. Fields were selected based on high *M. hapla* and *Pratylenchus* spp. population densities in pre-study surveys. Fields were located in western and central New York State, and planted with white-skinned potato cultivars for the fresh and chipping markets (cvs. Eva, Lamoka, and Genessee; Table 4.1). Due to a mixed population of *P. penetrans* (pathogenic to potato) and *P. crenatus* (cereal root-lesion nematode; non-pathogenic to potato) in Field 3 in 2016, another field was chosen in 2017 (Field 4; Table 4.1).

Table 4.1. Metadata for characterization of spatial and spatiotemporal attributes of Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.) populations in three commercial potato fields in western and central New York State in 2016 and 2017.

Year	Field	GPS Coordinates	Soil Classification[a]	Potato Cultivar	Initial Sampling	Final Sampling	Distance between samples (m)
2016	1	42.51° N, -79.77° W	Chenango gravelly loam	Eva	17 May	30 Aug	15.2 × 15.2
	2	42.50° N, -78.77° W	Chenango gravelly loam	Eva	17 May	30 Aug	4.6 × 12.2
	3	42.60° N, -77.51° W	Bath channery silt loam	Lamoka	25 May	15 Sept	4.6 × 6.1
2017	1	42.51° N, -79.77° W	Chenango gravelly loam	Genesee	1 June	6 Sept	15.2 × 15.2
	2	42.50° N, -78.77° W	Chenango gravelly loam	Genesee	1 June	6 Sept	4.6 × 12.2
	4	42.51° N, -78.76° W	Chenango gravelly loam	Eva	1 June	5 Sept	15.2 × 15.2

[a] United States Department of Agriculture – Natural Resources Conservation Service – Web Soil Survey.

Soil samples were collected on a 10 × 10 grid in each field, with distances between sampling locations within fields ranging from 4.6 to 15.2 m (Table 4.1). Soil samples were collected after planting (initial) and prior to harvest (final) in each year and locations were geospatially referenced using a Garmin GPS 72H unit (Garmin International Inc, Olathe, KS) at the initial sampling time (Table 4.1). At each sampling, soil (approximately 1 kg) was gathered from the hill along a length of 1 m at each grid point using a hand trowel to a depth of 15 cm, and stored separately in labeled plastic bags. Individual samples were homogenized by inverting the plastic bag several times, and 200 g subsamples were removed for manual nematode extraction and quantification through a modified Whitehead tray method (Whitehead and Hemming 1965; Gorny et al. 2018). Briefly, the soil was placed on top of a milk filter (KenAG, Ashland, OH) and single-ply facial tissue (Kleenex; Kimberly-Clark Corp., Irving, TX) resting on a mesh support, nested inside a 25.4 cm diameter pastry baking pan. Approximately 300 ml of distilled water was added to the lowest pan. Soil was incubated at room temperature in the dark for 48 h, after which nematodes were collected by passing the water from the lower pan twice through a moistened 25 µm mesh sieve held at a 45° angle. Collected nematode suspensions were allowed to settle for 24 h, adjusted to 25 ml, and 5 ml removed for population quantification under 400× magnification. *M. hapla* is the only root-knot nematode species present in New York State (Mitkowski et al. 2002), therefore all root-knot nematode second-stage juveniles (J2s) observed were recorded as *M. hapla*. Additionally all vermiform stages of *Pratylenchus* spp. were tallied. Counted populations were transformed to represent numbers within the 200 g subsample. Soil samples were stored at 4°C for up to 7 days before extraction. Nematode extraction efficiency of the modified Whitehead tray method was approximately 45% (A. Gorny, unpublished data).

Semivariogram estimation and modeling. Geostatistics are a subset of statistical methods used to analyze regionalized variables distributed in space and quantify associated uncertainty (Chilès and Delfiner 2009) and may be used to assess spatial patterns of nematode populations and quantify the degree of spatial autocorrelation (spatial dependence). In the case of spatial autocorrelation, locations closer together are likely to be more similar in characteristics than locations farther apart (Cressie 1989). Geostatistics can also predict or estimate values at non-sampled locations through weighted interpolations (Cressie 1989; Nelson et al. 1999).

Nematode population density data were log(x+1) transformed prior to semivariogram analysis and kriging to normalize probability distributions. Semivariograms are plots of one-half variance (semivariance) of paired samples by the distance between samples, or lags (Matheron 1963; Chilès and Delfiner 2009), and used to visualize the degree of spatial autocorrelation of *M. hapla* and *Pratylenchus* spp. populations. If samples are spatially autocorrelated, locations separated by a smaller distance will tend to have lower semivariance than locations separated by greater distances. The candidate semivariograms were fitted with various functions to smooth and estimate variogram parameters (Franklin et al. 2002). The sill was defined as the value at which the semivariance plateaus. The range was defined as the distance at which the semivariance reaches the sill and represents the smallest distance over which the nematode populations exhibited no spatial dependence. At a separation distance of zero, the corresponding calculated semivariance may not be zero due to spatial heterogeneity, measurement error from extraction efficiency, variance between replicate extractions, or size of subsample (Wallace and Hawkins 1994). This phenomenon was evaluated by the nugget, which quantifies the degree of dissimilarity expected in samples collected from extremely near locations (Cressie 1988).

Semivariogram modeling was performed in R (v. 3.3.3; R Core Team). *M. hapla* and *Pratylenchus* spp. population count data for each field × sampling interval × nematode species was assessed, and semivariograms constructed using the function 'variogram' in the R package 'gstat' (Pebesma 2004; Gräler et al. 2016). To determine the appropriate number of distance bins, Sturge's rule was applied (Legendre and Legendre 1998), which states K = 1 + 3.3 log(N); K = number of bins, and N = number of observations in the set (herein = 4,950). This yielded approximately 13 distance bins. Then, several semivariogram models (exponential, Gaussian, linear, logarithmic, Matern, nugget, spherical, and wave) were tested for their fit using the function 'fit.variogram' in the R package 'gstat', and the best fit model (smallest residual sum of squares) was chosen. The range, sill, and nugget of each best fit model was recorded. The relative structure, or the proportion of the sample variance captured by the best fit model was also calculated as the ratio of structural variance (difference between the sill and the nugget, C) to sample variance (structural variance plus the sill, $C + C_0$) (Robertson and Freckman 1995; Ettema et al. 1998; Ettema and Wardle 2002). The relative structure is a measure of spatial dependence, with values varying from 0 (no spatial dependence) to 1 (perfect spatial dependence) (Robertson and Freckman 1995).

Ordinary kriging. Ordinary kriging was conducted to interpolate and estimate values between sampled locations using a function of weighted averages of known values defined by the optimal semivariogram model (Cressie 1988; Wallace and Hawkins 1994; Chilès and Delfiner 2012). Weights were calculated to minimize variance, and smooth the predicted values across the study area (Wallace and Hawkins 1994; Haining 2003; Chilès and Delfiner 2012). Kriging was performed in R with the function 'krige' (package 'gstat'; Pebesma 2004), using parameters from the semivariogram best fit model and a 3 m × 3 m interpolation grid for each field × sampling interval × nematode species combination. Interpolation maps of the resultant predictions were visualized

using the function ‘spplot’ (package ‘sp’; Pebesma and Bivand 2005; Bivand et al. 2013).

Spatial Analysis by Distance IndicEs. Spatial Analysis by Distance IndicEs (SADIE) is a method for calculating aggregation indices for quantifying spatial patterns (Perry 1995). The SADIE method is complementary to semivariogram and ordinary kriging in that it is specialized for assessing spatial patterns and useful when individuals or values are discretely aggregated into approximate boundary-defined patches, whereas geostatistical methods tend to overly smooth values across the measured space (Winder et al. 2001).

Spatial and spatiotemporal patterns of *M. hapla* and *Pratylenchus* spp. populations were explored using SADIE (v. 1.22). For count data with local spatially referenced coordinates, SADIE uses a transportation algorithm to determine the minimum number of moves in which the individuals must move in the space so that all sampling units have equal or nearly equal numbers of individuals (Perry 1995; 1998). The moves are summed to give the “distance to regularity” D, and the calculation repeated with sets of permuted, randomized data, E_a. The ratio of the observed to the permutated results is the index of aggregation, I_a, a measure of the overall departure from randomness and defined as $I_a = D/E_a$ (Perry 1995; 1998). Values significant for $I_a > 1$ are derived from aggregated or clustered data, while those values significant for $I_a < 1$ are suggestive of regular patterns. Values in which $I_a = 1$ are derived from randomly distributed data (Perry 1995; 1998). The null hypothesis is a random spatial distribution. For all SADIE simulations, 5,967 randomizations were used and the same seed (12,345) was called.

Associations in nematode populations between the initial and final sampling times were explored using the Association Function of SADIE (v. 1.22) by comparing clustering indices generated from two consecutive sampling periods in time (Winder et al. 2001). The Association Function of SADIE was also used to quantify co-occurrence

between *M. hapla* and *Pratylenchus* spp. populations at both samplings within fields. The overall association (*X*) was evaluated by calculating the correlation coefficient between the clustering indices generated from SADIE analysis at either sampling (Pethybridge and Turechek 2003; Winder et al. 2001). The null hypotheses were no spatiotemporal association between sampling intervals or between *M. hapla* and *Pratylenchus* spp. populations at each sampling. For all association simulations, a total of 9,999 randomizations were used and the same seed (12,345) was called.

Results

Semivariogram estimation and modeling. Nematode populations were spatially heterogeneous in both years. Mean values for *M. hapla* populations were 48 to 115 J2s at the initial sampling, and 38 to 3,511 J2s at the final sampling (Table 4.2). Mean values for *Pratylenchus* spp. populations were 28 to 931 at the initial sampling, and 27 to 1,547 at the final sampling (Table 4.2). Semivariogram analysis suggested minor to strong degrees of aggregation for *M. hapla* and *Pratylenchus* spp. populations in all fields indicating spatial dependence at both samplings. The semivariogram models providing the best fit included linear, spherical, Gaussian, exponential, and logarithmic. A pure nugget effect was not detected for either nematode population (Table 4.3). Population variances were approximately 13 to 100% spatially structured among *M. hapla*, and 7 to 100% spatially structured among *Pratylenchus* spp. populations, with 4,950 interpolation points per analysis (Table 4.3). Distances over which *M. hapla* populations were spatially autocorrelated were 18.7 to 232.6 m (mean (μ) = 110.3 m) at the initial sampling and 69.3 to 277 m (μ = 149.3 m) at the final sampling. *Pratylenchus* spp. populations were correlated at the initial sampling over 42.5 to 378.2 m (μ = 146.6 m) and at the final sampling over 48.7 to 315.4 m (μ = 164.9 m) (Table 4.3).

Table 4.2. Average populations per 200 g of soil and statistical measures of variability of the Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.) populations at the initial and final samplings in three commercial potato fields in western and central New York State in 2016 and 2017.

Species	Field	Year	Sampling Interval	Mean	SD[a]	CV[b]	Range (Min – Max)
Meloidogyne hapla	1	2016	Initial	73	180	248	0 – 1,038
			Final	2,116	3,758	178	0 – 20,587
		2017	Initial	48	65	134	0 – 344
			Final	221	485	219	0 – 2,550
	2	2016	Initial	115	178	154	0 – 1,588
			Final	3,511	3,889	111	0 – 23,338
		2017	Initial	52	88	170	0 – 544
			Final	299	933	312	0 – 7,742
	3	2016	Initial	51	153	297	0 – 1,438
			Final	38	95	253	0 – 588
	4	2017	Initial	53	106	198	0 – 781
			Final	466	908	195	0 – 6,208
Pratylenchus spp.	1	2016	Initial	77	103	134	0 – 506
			Final	82	154	189	0 – 788
		2017	Initial	33	34	103	0 – 150
			Final	27	37	137	0 – 142
	2	2016	Initial	72	106	148	0 – 554
			Final	58	115	199	0 – 999
		2017	Initial	28	37	132	0 – 194
			Final	27	46	167	0 – 225
	3	2016	Initial	931	484	52	94 – 2,275
			Final	1,547	1,213	78	75 – 5,588
	4	2017	Initial	98	93	95	0 – 456
			Final	78	112	143	0 – 833

[a] SD = standard deviation.
[b] CV = coefficient of variation, %.

Table 4.3. Parameters of fitted semivariogram models for assessment of spatial dependence of Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.) populations in three commercial potato fields in western and central New York State in 2016 and 2017.

Species	Field	Year	Sampling Interval	Model	Relative structure ($C/(C + C_0)$)	Nugget (C_0)	Range (m)	Sill ($C + C_0$)
Meloidogyne hapla	1	2016	Initial	Linear	0.447	0.368	116.7	0.667
			Final	Matern	0.340	0.842	132.8	1.275
		2017	Initial	Linear	0.423	0.265	125.9	0.459
			Final	Linear	0.178	0.958	186.2	1.165
	2	2016	Initial	Linear	0.203	0.349	53.0	0.438
			Final	Wave	0.130	0.699	122.8	0.804
		2017	Initial	Linear	0.788	0.160	114.6	0.755
			Final	Spherical	0.629	0.560	107.6	1.508
	3	2016	Initial	Matern	1.0	0.00	18.7	0.425
			Final	Wave	0.227	0.612	69.3	0.792
	4	2017	Initial	Linear	0.676	0.213	232.6	0.657
			Final	Matern	0.841	0.317	277.0	1.997
Pratylenchus spp.	1	2016	Initial	Spherical	0.815	0.103	126.1	0.557
			Final	Matern	0.768	0.141	48.7	0.609
		2017	Initial	Gaussian	1.0	0.00	42.5	0.343
			Final	Exponential	0.478	0.382	315.4	0.732
	2	2016	Initial	Linear	0.267	0.446	57.2	0.609
			Final	Linear	0.303	0.567	190.7	0.814
		2017	Initial	Linear	0.296	0.333	174.2	0.473
			Final	Linear	0.449	0.337	62.4	0.612
	3	2016	Initial	Gaussian	0.257	0.055	101.1	0.074
			Final	Logarithmic	0.070	0.107	62.4	0.115
	4	2017	Initial	Linear	0.465	0.153	378.2	0.286
			Final	Linear	0.465	0.373	309.7	0.497

Ordinary kriging. Interpolation maps of *M. hapla* and *Pratylenchus* spp. populations were produced by ordinary kriging (Fig. 4.1). By visual inspection, areas of predicted high *M. hapla* populations were not always spatially associated with areas of predicted high *Pratylenchus* spp. populations.

Spatial Analysis by Distance IndicEs. Significant aggregation of *M. hapla* populations was detected in only Field 1 at the final sampling in 2016, and Field 4 at both sampling times in 2017 ($P \leq 0.03$; Table 4.4). *Pratylenchus* spp. populations were significantly aggregated ($P \leq 0.04$) in nearly all fields at both times (10 of 12 samplings), with the exception of Field 2 at the initial sampling in 2016, and Field 3 at the final sampling in 2016 (Table 4.4). Significant spatiotemporal associations were detected between the initial and final samplings for *M. hapla* and *Pratylenchus* spp. in 2016 and 2017 ($P \leq 0.01$; Table 4.4) with the exception of Field 1 in 2016 due to an inability to calculate *D*, and Field 3 in 2016 for *Pratylenchus* spp. populations ($P > 0.05$; Table 4.4). There was significant co-occurrence between *M. hapla* and *Pratylenchus* spp. populations in 6 of 12 samplings ($P \leq 0.049$; Table 4.5).

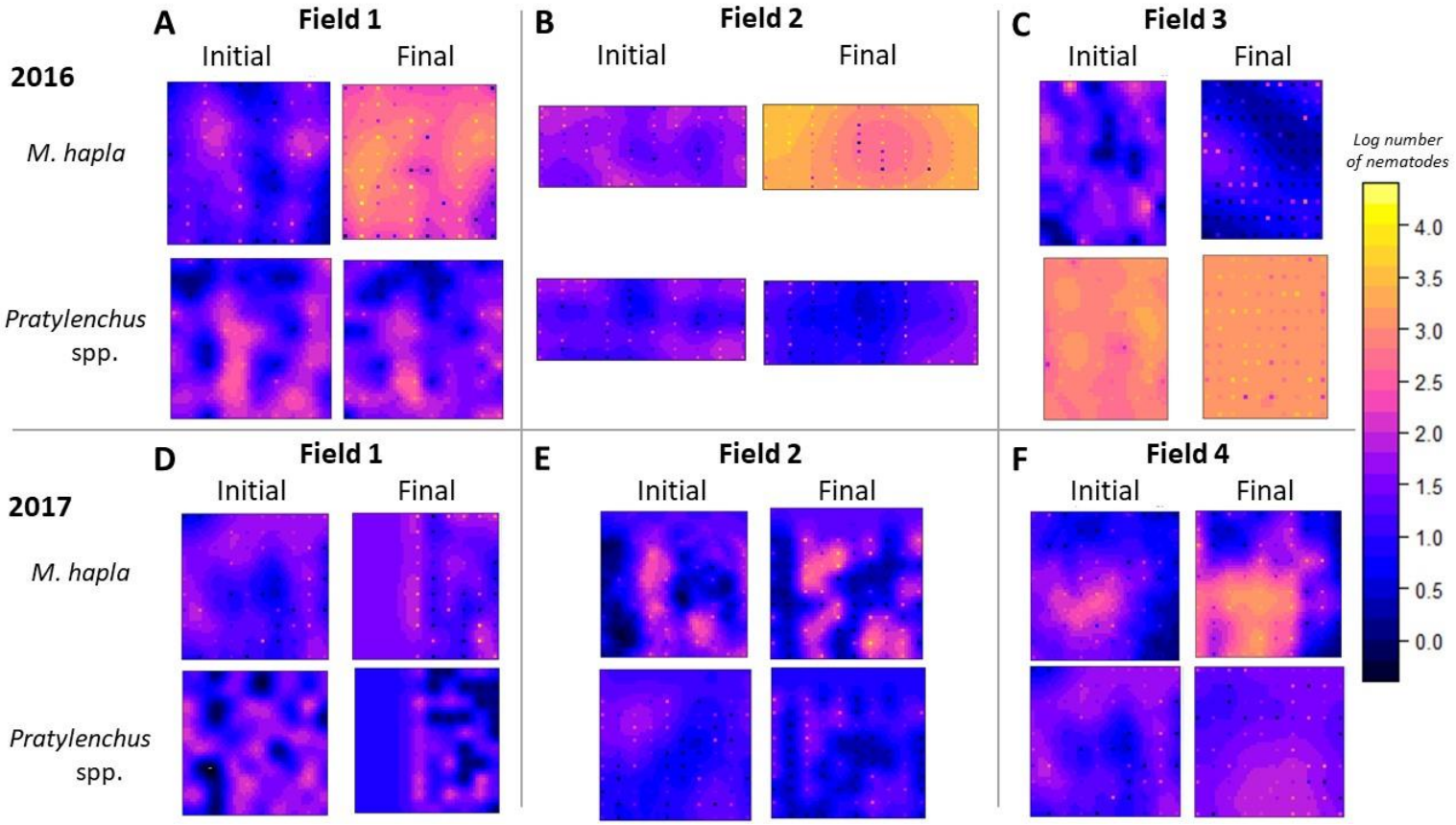

Fig. 4.1. Three commercial potato fields were sampled in each of 2016 and 2017 for populations of the Northern root-knot nematode, *Meloidogyne hapla*, and root-lesion nematode, *Pratylenchus* spp. Nematode population densities were determined from sampling locations using a manual extraction procedure, and interpolation plots produced through semivariogram analysis and ordinary kriging. Soil samples were collected after planting (initial) and prior to harvest (final) for Fields 1 (**A**), 2 (**B**), and 3 (**C**) in 2016, and Fields 1 (**D**), 2 (**E**), and 4 (**F**) in 2017. Marks in the scale represent log increase in number of nematodes.

Table 4.4. Summary of statistics produced from Spatial Analysis by Distance IndicEs (SADIE) and Association Function of SADIE for analysis of within field aggregation for Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.) populations in three commercial potato fields in New York State in 2016 and 2017.

Species	Field	Year	Sampling Interval	D^a	I_a^b	P^c	X^d (P =)
Meloidogyne hapla	1	2016	Initial	158,923	0.982	0.4853	*N/A*
			Final	5,025,637	1.318	0.0292	
		2017	Initial	65,609	1.033	0.3553	0.241
			Final	421,318	1.185	0.1351	(0.0137)
	2	2016	Initial	129,800	1.389	0.7090	0.406
			Final	2,321,206	1.294	0.1354	(0.0001)
		2017	Initial	95,704	1.096	0.2425	0.434
			Final	798,893	0.972	0.4977	(< 0.0001)
	3	2016	Initial	50,259	1.106	0.2524	0.252
			Final	29,261	0.929	0.6077	(0.0123)
	4	2017	Initial	152,585	1.508	0.0059	0.546
			Final	1,525,696	1.738	0.0050	(< 0.0001)
Pratylenchus spp.	1	2016	Initial	152,545	1.475	0.0094	*N/A*
			Final	226,047	1.548	0.0059	
		2017	Initial	46,751	1.367	0.0253	0.311
			Final	36,410	1.642	0.0070	(0.0104)
	2	2016	Initial	80,665	1.415	0.0662	0.709
			Final	89,096	1.501	0.0449	(< 0.0001)
		2017	Initial	61,110	1.673	0.0003	0.597
			Final	71,412	1.565	0.0039	(< 0.0001)
	3	2016	Initial	3,535,146	2.034	0.0002	-0.210
			Final	526,288	1.203	0.1111	(0.9759)
	4	2017	Initial	197,293	2.113	0.0002	0.589
			Final	179,560	1.681	0.0012	(< 0.0001)

[a] Distance to regularity.
[b] Index of Aggregation.
[c] Significance value for the Index of Aggregation.
[d] Overall association and significant value from the Association Function of SADIE.

Table 4.5. Summary of statistics produced from the Association Function of Spatial Analysis by Distance IndicEs for assessment of co-occurrence of Northern root-knot nematode (*Meloidogyne hapla*) and root-lesion nematode (*Pratylenchus* spp.) populations in three commercial potato fields in New York State in 2016 and 2017.

Field	Year	Sampling Intervals	X [a]	P =
1	2016	Initial	0.1556	0.092
		Final	0.1001	0.049
	2017	Initial	- 0.1005	0.847
		Final	0.2956	0.014
2	2016	Initial	0.0793	0.225
		Final	0.2505	0.008
	2017	Initial	0.0663	0.302
		Final	0.2238	0.207
3	2016	Initial	0.2364	0.01
		Final	0.0119	0.462
4	2017	Initial	0.2274	0.017
		Final	0.2200	0.012

[a] Overall association from the Association Function of SADIE.

Discussion

Three commercial potato fields were intensively sampled in each of two years, 2016 and 2017, to better understand spatial and spatiotemporal patterns of *M. hapla* and *Pratylenchus* spp. The spatially heterogeneous *M. hapla* and *Pratylenchus* spp. populations described in this study were consistent with findings for other soilborne plant-parasitic nematodes (Goodell and Ferris 1980; Wallace and Hawkins 1994; Farias et al. 2002; King and Taberna 2013). In the present study, a strong right-hand skewness was observed in nematode populations, with a high incidence of zero or low (≤ 100 individuals / 200 g soil) *M. hapla* and *Pratylenchus* spp. populations in all fields at both samplings. This distribution has also been observed in other studies, for both plant-parasitic and non-parasitic nematode taxa (Robertson and Freckman 1995; Goodell and Ferris 1980; Ferris et al. 1990). The means and variances of the nematode populations measured in this study were similar to those described by Wheeler et al. (1994) for initial densities of *M. hapla* (approximate difference of –2.5%), and higher than those for *Pratylenchus* spp. (approximate difference of +1,490%).

Geostatistical methods determined that *M. hapla* and *Pratylenchus* spp. populations were spatially autocorrelated in every field sampled. Robertson and Freckman (1995) concluded that plant-parasitic nematodes were not spatially autocorrelated in a study assessing spatial distributions of different nematode trophic groups, reporting a pure nugget effect in the semivariogram constructed for plant-parasitic nematodes. However, unlike the other taxonomic groups evaluated, plant-parasitic nematodes are obligate biotrophs, with differing species of host plants supporting different spectra of plant-parasitic nematodes. The spatial distribution of potential host plants could have therefore influenced the arrangement of plant-parasitic nematodes. The study did not further segment the group into lower taxa (such as genera or species), and consequently

any spatial dependencies were potentially veiled due to the bulking of diverse plant-parasitic nematode taxa into one group.

Wallace and Hawkins (1994) found the species measured in their study (including *P. penetrans, Tylenchorhynchus* spp., *Heterodera trifolii, Paratylenchus* spp., and *Criconemella* spp.) were spatially autocorrelated over 40 to 90 m, and an additional population of *Aglenchus agricola* at 160 m. Moreover, Evans et al. (2002) generalizes that nematode populations are spatially autocorrelated over 40 to 60 m. Here we found *M. hapla* populations were correlated at the initial sampling over a range of 18.7 to 232.6 m (μ = 110.3 m) and at the final sampling over 69.3 to 277 m (μ = 149.3 m). *Pratylenchus* spp. populations were correlated at the initial sampling over 42.5 to 378.2 m (μ = 146.6 m) and at the final sampling over 48.7 to 315.4 m (μ = 164.9 m). The range represents the maximum distance at which spatial dependence can be expected (Wallace and Hawkins 1994; Chilès and Delfiner 2009). Sample spacing wider than the range will not contribute to discovery of spatial dependence, while spacing that does not reach this will leave part of the spatial structure undiscovered (i.e. the sill) (Webster and Oliver 2007). Therefore, based on our findings, we recommend approximately 110 m and 147 m sample spacing for assessment of initial *M. hapla* and *Pratylenchus* spp. populations, respectively. Autocorrelation ranges within the same field were between 12.1 to 86.5% shorter at the initial than at the final sampling times for both nematode species in 7 of 12 comparisons. This difference in ranges could be explained by expansion of disease foci through migration of nematodes horizontally through the soil, or dispersal on machinery or surface water.

Although geostatistical analyses indicated *M. hapla* populations were aggregated, SADIE indicated a random spatial distribution within most fields sampled. Within the SADIE method, the position of the individual count data within the sampling area is important because the distance to regularity measures the total moves required to

distribute counts evenly to all spatial units within the sampling area (Perry 1995). Therefore, high populations occurring in the center of the sampling area would be calculated as less aggregated than high populations occurring near the edges (Perry 1995). This approach is not utilized in the geostatistical method, and could account for the difference in assignment of significant aggregation.

Spatiotemporal analysis of *M. hapla* and *Pratylenchus* spp. populations within fields between samplings provided evidence for significant association in nearly all cases. This suggests that the location of nematode populations remained relatively constant through the season. The exception was *Pratylenchus* spp. populations in Field 3 in 2016. This may be explained by the presence of a mixed population of *P. penetrans* and *P. crenatus* within Field 3, which were not differentiated during manual counting in our study.

Ferris et al. (1990) posits that sedentary endoparasites (e.g., *M. hapla*) are typically observed in aggregated patterns because eggs are laid in clusters immediately around a stationary female. Conversely, migratory endo- and ectoparasites (including species in the *Pratylenchus* genus) are likely to exhibit a more disperse or random patterning because eggs are laid as females migrate through the soil or host plant tissues (Ferris et al. 1990). In this study, geostatistical methods suggested both *M. hapla* and *Pratylenchus* spp. were aggregated, while SADIE observed that *Pratylenchus* spp. populations were aggregated at a higher frequency than *M. hapla*. This finding is contrary to that predicted by Ferris et al. (1990), potentially indicating that other factors (such as field topography, edaphic factors, movement of farm equipment, or microclimates within the field) in addition to nematode biology may have a dominating influence on spatial patterns.

Enhanced knowledge of spatial patterns of nematode populations can also be used to explore the potential for site-specific nematicide application (Evans et al. 2002; King

and Taberna 2013). As populations are spatially heterogeneous within fields, uniform application of nematicides have a high probability of over and under optimal use and/or rates in some areas (Evans et al. 2002; Mueller et al. 2010). Future research clarifying the association between *M. hapla* and *Pratylenchus* spp. populations and crop loss would allow for the calculation of economic thresholds and development of crop loss models to support a rigorous cost-benefit analysis for site-specific nematicide application or other control methods. The accuracy of economic thresholds and crop loss models may be refined by additional research into the complex relationship of edaphic factors such as soil texture, pH, temperature, and nutrient levels on crop loss and nematode populations (Melakeberhan and Avendaño 2008). For example, Liu et al. (2014) observed that populations of the Southern root-knot nematode (*M. incognita*) at planting significantly influenced yield in cotton, but the economic threshold was significantly influenced by soil texture (percent sand content). Soil texture has also been assessed for suitability as a proxy for estimating nematode populations, crop loss, and returns from nematicide applications (Avendaño et al. 2004; Monfort et al. 2007; Kulkarni et al. 2008; Liu et al. 2014).

When considering the implementation of site-specific nematicide application, a spatially heterogeneous, but highly structured nematode populations within fields is essential (Avendaño et al. 2004; Melakeberhan and Avendaño 2008). Populations encountered of *M. hapla* and *Pratylenchus* spp. had broad range of values for relative structure (13 to 100% for *M. hapla*, and 7 to 100% for *Pratylenchus* spp.), dependent upon the field and the sampling interval. This finding suggests that site-specific nematicide application would only be practical in only a few cases. Furthermore, when considering site-specific application for dual control of both *M. hapla* and *Pratylenchus* spp. in commercial potato fields, it is necessary that the spatial patterns of the populations should be overlapping. The co-occurrence of *M. hapla* and *Pratylenchus*

spp. in only half of the samplings negates the potential for this approach in many of these fields. However, further investigations determining the economic impact of either nematode individually or together on potato production would facilitate these decisions.

Hindrance to widespread adoption of spatial analysis of pre-plant nematode populations has resided in the cost of sampling and mapping spatially heterogeneous populations in the field (Mueller et al. 2010) and in the analysis and precision of the spatial data generated (Anselin et al. 2004; Liu et al. 2014). The ability to ensure nematode sampling remains economically viable lies in balancing the cost of collecting and processing soil samples, and the ability to generate spatially explicit maps to guide management such as site-specific nematicide application. A finer sampling scale or an increased number of samples will result in a more precise map, but will also increase the cost of analysis. The knowledge gained in this study aids in the goal of economically and environmentally sustainable nematode management by providing data on optimal sample spacing and intensity based on spatial patterns of *M. hapla* and *Pratylenchus* spp. populations in commercial potato fields in New York State.

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CHAPTER 5

EVALUATING THE ECONOMIC THRESHOLD FOR THE NORTHERN ROOT-KNOT NEMATODE (*MELOIDOGYNE HAPLA*) IN NEW YORK STATE POTATO PRODUCTION

Abstract

In New York State and the Northeastern United States, the Northern root-knot nematode (RKN), *Meloidogyne hapla*, is an important soilborne pathogen of potato and other vegetable crops. The economic threshold for *M. hapla* in potato production in New York State has been 100 second-stage juveniles (J2s) per 100 g of soil, and is often used to dictate the need for pre-plant nematicides. However, changes in popular cultivars and potentially susceptibility has warranted a reevaluation of this threshold. The relationships between initial *M. hapla* population densities and potato yield components in the popular processing and fresh market cvs. Envol, Eva, and Lamoka, were evaluated in three commercial fields in western and central New York State in 2016 and 2018. *M. hapla* population densities were quantified from soil collected at multiple locations (*n* = 100) within three sampling grids per year, following manual extraction with a modified Whitehead tray. Moreover, a nested quantitative PCR (qPCR) assay was developed for estimating *M. hapla* populations directly from soil as an alternative to morphology-based nematode identification and quantification. The relationship between *M. hapla* populations estimated through manual extraction and qPCR, and potato yield and damage was quantified. Initial *M. hapla* populations quantified through manual extraction and the qPCR assay were positively correlated in both years ($r = 0.47$ and 0.33; $P < 0.0001$). No significant association was found between the initial *M. hapla* populations quantified through either method and total yield, tuber number, average

tuber diameter, or galling severity on tubers. Results suggest these cultivars may be more tolerant to *M. hapla* and the economic threshold may be higher than currently used. Additionally, these findings suggest that prophylactic use of pre-plant nematicides for control of *M. hapla* in these potato cultivars in New York State may not be warranted.

Introduction

Potatoes are an important vegetable crop in New York State and the Northeastern United States. In 2018, New York State produced approximately 5,900 hectares of potatoes for the fresh market and chipping industries (USDA-NASS 2019). Potatoes are host to several species of plant-parasitic nematodes, including the Northern root-knot nematode (RKN), *Meloidogyne hapla* (Brodie et al. 1993; Perry et al. 2009). *M. hapla* is the dominant RKN species in the typical temperate, intensive, broadacre rotations in New York State (Mitkowski et al. 2002), and is particularly damaging to carrot (Gugino et al. 2006), tomato (Barker et al. 1976), and lettuce (Viaene and Abawi 1996). In potato, infection and feeding by *M. hapla* may reduce yield, and deleteriously affect quality by causing aesthetic damage to tubers through development of galls, swelling, and blemishes on the surface (Vovlas et al. 2005). Several types of nematicides are currently available for use on potato in New York State, including fumigant (e.g., 1,3-dichloropropene), non-fumigant (e.g., oxamyl) and biological materials. The former are relatively rare in vegetable production in New York State due to the difficulties associated with penetration of the profile in heavy clay, mineral soils (Gugino et al. 2006). Crop rotation is another method frequently used for control of plant-parasitic nematodes. However, *M. hapla* is difficult to effectively control through crop rotation due to its polyphagous host range (Duncan 1991).

Initial nematode populations (i.e., those present early in the growing season) are often inversely correlated with crop loss (Seinhorst 1965; Barker and Olthof 1976; Madden et al. 2007). For example, LaMondia (1995) described a significant reduction in tobacco leaf yield as populations of the tobacco cyst nematode *Globodera tobacum tobacum* increased, with initial population densities of 50 to 100 second-stage juveniles (J2s) per 100 cm^3 of soil resulting in a 1 to 5% yield reduction. Further, yield reductions increased exponentially when initial population densities ranged between 100 and 300 J2s per 100

cm^3 of soil (LaMondia 1995). In potato, Olthof and Potter (1972) observed a yield reduction of 46% in the potato cv. Sebago in microplots infected with 18,000 *M. hapla* J2s per kg soil when compared to non-infested plots. Stirling and Wachtel (1985) also described significant yield reductions in Australian potato fields infested with *M. hapla*, and noted that yield was increased when fumigant nematicides were applied prior to planting.

To mitigate yield loss in high value potato and vegetable crops, producers in New York State and the Northeastern United States may prophylactically apply nematicides prior to planting for the control of *M. hapla* and other plant-parasitic nematodes (Schmitt and Sipes 2002; Gugino et al. 2006). However, if undertaken without evidence of nematode population densities, this tactic may potentially result in false-positive decisions, in which nematicides are applied when initial populations are below the economic threshold level, defined as the initial pest or pathogen population to make implementation of control tactics economically viable (Stern et al. 1959). These false-positive decisions may lead to less desirable outcomes, including off-target environmental impacts and increased cost of production without pest control justification (Hay et al. 2016).

Earlier research identified 100 *M. hapla* J2s per 100 g of soil as the economic threshold for *M. hapla* in potato based on research conducted on the cv. Sebago (Olthof and Potter 1972; Barker and Olthof 1976). Therefore, when *M. hapla* population densities are above this threshold, a corresponding reduction in yield valued at greater than the cost of control may occur, thus warranting nematicide application or other control, such as crop rotation to a non-host (Stern et al. 1959; Southwood and Norton 1973). Although informative, these studies used older potato cultivars that have fallen out of favor, and the economic threshold for *M. hapla* in newer potato cultivars preferred by producers in New York State remains largely unknown. An improved knowledge of

economic thresholds pertinent to local conditions and current potato cultivars may therefore provide New York State producers a foundation for making decisions on when it is economically prudent to undertake certain management or control operations for soilborne pathogens such as *M. hapla*.

Quantification of nematode population densities by extraction and counting from soil is the standard method to evaluate their density compared to the economic threshold. These methods involve manual extraction of live, motile nematodes through techniques such as Baermann funnels (Baermann 1917), Whitehead trays (Whitehead and Hemming 1965), elutriation (Seinhorst 1956), or density gradient flotation (Viglierchio and Yamashita 1983), followed by morphology-based identification and quantification under high power magnification. Although useful, these standard manual extraction methods are time consuming and frequently have low extraction efficiencies, potentially underestimating true population densities (Hay et al. 2016). Further, identification requires advanced training in nematode morphology and may often only be made to the genus level (Sawada et al. 2011; Min et al. 2012; Sapkota et al. 2016).

The use of DNA-based molecular assays for pathogen detection and quantification offers several advantages over traditional methods, including reduced reliance on morphology-based identification, suitability for high throughput analysis, and the potential to test for multiple pathogens in a single assay. For the detection and quantification of RKN and *M. hapla* populations, assays based on quantitative real-time PCR (qPCR; e.g., Hay et al. 2016; Sapkota et al. 2016; Gorny et al. 2019), loop mediated isothermal amplification (Niu et al. 2001; Peng et al. 2017), or sequence-characterized amplified regions (Zijlstra 2000) have been described. The use of DNA-based molecular assays for detection and quantification of soilborne pathogens (including plant-parasitic nematodes) has also been translated into support schemes for practical pathogen management decisions in commercial production, such as the PredictaPt (for potato)

and PredictaB (for broad acre crops) tests provided by the South Australian Research & Development Institute (SARDI) (Ophel-Keller et al. 2008). Defining a new economic threshold for *M. hapla* in potato production using a DNA-based molecular assay may also facilitate more rapid, accurate and precise evaluations of populations.

The objectives of this study were to: (i) quantify the accuracy of a nested qPCR assay for detection of *M. hapla* directly from soil compared to the standard method of manual extraction and quantification, and (ii) quantify the association between initial *M. hapla* populations and potato yield components in selected popular cultivars in New York State. This information will evaluate the economic threshold of 100 *M. hapla* J2s per 100 g of soil in potato production currently used by producers to define the need for pre-plant nematicide applications.

Materials and Methods

Soil sampling and yield data collection. Commercial potato fields with elevated natural populations of *M. hapla* were intensively sampled in 2016 and 2018. In 2016, three separate fields were included with one gridded sampling area in each field. In 2018, one field was selected, and three sampling grids were placed in different sections of the same field. Fields (approximately 3 to 10 ha in size; Table 5.1) were located in western and central New York State, and planted with white-skinned potato cultivars popular for the fresh market and chipping industries (cvs. Envol (Anonymous 2013), Eva (Plaisted et al. 2001), and Lamoka (De Jong et al. 2017); Table 5.1). Samples were collected in a 10 × 10 grid matrix, with distances between locations ranging from 4.6 to 15.2 m in 2016, and 1 to 5 m in 2018 (Table 5.1). Sampling locations were geospatially referenced with a Garmin 72H GPS unit (Garmin International Inc., Olathe, KS) and marked with a flag at the initial sampling. Initial *M. hapla* population densities (P_i) were measured by collecting soil at the sampling locations just after planting (17 and 25 May

2016; 14 May 2018; Table 5.1). Using a hand trowel, approximately 1 kg of soil was collected from the row hills along a 1 m transect to a depth of 15 cm. Final *M. hapla* population densities (P_f) were measured by sampling the same locations prior to harvest (30 August and 15 September 2016; 9 and 10 August 2018) using the same procedure. Yield at each point was also quantified at this sampling by collecting all potato tubers from the 1 m transect. Tubers were collected into 7.6 L plastic zip-lock bags, returned to the laboratory and stored for up to 3 days at 4°C prior to assessment. Tubers within each sampling location were weighed (kg) and counted. The long axis diameter of each tuber (mm) was measured using a digital hand caliper (Electron Microscopy Sciences, Hatfield, PA). Tubers were also evaluated for cosmetic damage due to *M. hapla* using a modified version of the powdery scab severity scale of Baldwin et al. (2008). Tuber galling severity was scored at the sampling location level on an ordinal scale from 0 (no galls) to 6 (all tubers covered in galls).

Table 5.1. Meta-data for commercial potato fields in New York State sampled for evaluation of the relationships between the Northern root-knot nematode (*Meloidogyne hapla*) populations in the soil and yield and quality in 2016 and 2018.

Year	Sampling grid	GPS coordinates	Cultivar	Initial sampling	Final sampling	Distance between samples (m)
2016	1	42.51° N -79.77° W	Eva	17 May	30 August	15.2 × 15.2
	2	42.50° N -78.77° W	Eva	17 May	30 August	4.6 × 12.2
	3	42.60° N -77.51° W	Lamoka	25 May	15 September	4.6 × 6.1
2018	1	42.51° N -78.76° W	Envol	14 May	9 August	1 × 5
	2	42.51° N -78.76° W	Envol	14 May	10 August	1 × 5
	3	42.51° N -78.76° W	Envol	14 May	10 August	1 × 5

Manual *M. hapla* extraction and quantification. Soil samples from the initial and final samplings were stored up to 7 days at 4°C before manual nematode extraction. Samples were homogenized by vigorously inverting the plastic bag several times, then removing a 200 g subsample from each for manual nematode extraction and quantification of P_i and P_f through a modified Whitehead tray method (Whitehead and Hemming 1965; Gorny et al. 2018). Briefly, a mesh support was nested inside a 25.4 cm diameter pastry baking tin. A milk filter (KenAG, Ashland, OH) and a single-ply facial tissue (Kleenex; Kimberly-Clark Corp., Irving, TX) were then placed over the mesh. The soil subsample was placed on top of the assembly, and approximately 300 ml of distilled water was added to the lower-most pan. The extraction assembly was incubated at room temperature in the dark for 48 h, after which nematodes were collected into individually labeled 50 ml falcon tubes by passing the water from the lower pan twice through a pre-wetted 25 µm mesh sieve held at 45°. Collected nematode suspensions were allowed to settle for 24 h at 4°C, then adjusted to 25 ml by carefully siphoning liquid off the top without disturbing nematodes at the bottom of the collection vial. An aliquot of 5 ml was removed for quantifying *M. hapla* populations under 400× magnification by counting the number of *M. hapla* J2s, then transforming counts to represent the total 200 g soil subsample. The extraction efficiency of the modified Whitehead tray method was approximately 45% (A. Gorny, unpublished data).

DNA extraction. Remaining soil (approximately 800 g) was air dried at room temperature for 10 days. *M. hapla* DNA was extracted from a 100 g subsample of this soil using the super paramagnetic iron oxide nanoparticle (SPION) capture method of Gorny et al. (2018) with minor modifications described here. Briefly, 100 g subsamples were placed into a 250 ml Nalgene bottle with screw cap lid and 50 ml of Lysis Buffer (100 mM Tris hydrochloride, 100 mM EDTA, 10 mM sodium chloride, 3% w/v enzymatic laundry detergent (Tide Original Powder Laundry Detergent, Procter and

Gamble, Cincinnati, OH), and 10 mg/ml RNase A (Qiagen Inc., Carlsbad, CA); pH 10.4) was added. Solutions were vortexed then heated in a water bath at 65°C for 2 h, after which they were vortexed at maximum speed for 30 sec on a VortexGenie fitted with a flat top attachment (VWR, Radnor, PA). Bottles were centrifuged at 5000 *g* for 10 min and supernatant transferred to a 50 ml falcon tube. To the supernatant, 10 mg Fe_3O_4 SPION suspended in sterile Type I water and 25 ml of Binding Buffer (20% w/v polyethylene glycol 8000, 4 M NaCl) were added. The mixture was incubated at room temperature for 20 min, with gentle inversion, after which SPION were immobilized using a magnetic rack, and supernatant discarded. SPION were washed three times with 70% ethanol and dried in sterile conditions for 2 h to remove trace ethanol. Bound DNA was then eluted from the SPION by washing with 200 µl of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.3) and incubating at room temperature for 5 min. SPION were immobilized with a magnetic rack and DNA solution transferred to a 1.5 ml tube. Resultant crude DNA extracts were purified using polyvinylpolypyrrolidone (PVPP) spin column. The PVPP spin column was assembled by nesting a 0.5 ml tube within a 2 ml tube, and a flame-sterilized needle tool was used to make a hole in the bottom of the smaller tube. A small quantity of sterile glass wool was compacted into the end over the hole. TE buffer was used to suspend PVPP (110 µm particle size, Sigma-Aldrich, St. Louis, MO) and pipetted onto the glass wool support. Column assemblies were centrifuged at 900 *g* for 1.5 min to pack the column and remove excess buffer. More PVPP solution was added and centrifugation repeated as needed until the column was 15 mm high. The completed PVPP column was then nested into a new 2 ml tube. One PVPP spin column was made for each DNA sample. Crude DNA from the SPION extraction was pipetted on top of the column, incubated for 10 min at room temperature, then centrifuged at 400 *g* for 1.5 min. Resultant DNA in the column flow-through was

quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C prior to use.

Detection and quantification of *M. hapla*. A nested qPCR assay was developed for estimation of *M. hapla* population densities from the soil DNA extracts. First, an initial PCR used the primer pair rDNA2 and rDNA1.58s to amplify a 433 bp region of the internal transcribed spacer 1 (ITS1) region (Powers et al. 1997). Reactions were run in duplicate and contained 1× standard PCR buffer with $MgCl_2$ (New England Biolabs, Ipswich, MA), 0.2 mM dNTPs (New England Biolabs), 8×10^{-4} mg bovine serum albumin (Fisher Scientific, Waltham, MA), 1% w/v skim milk powder (Walmart Inc., Bentonville, AR), 1.0 U standard *Taq* polymerase (New England Biolabs), 0.2 µM of each primer, 5 µl of template DNA, and sterile Type I water to bring the volume to 25 µl. The reaction was performed on a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). PCR conditions consisted of an initial denaturation of 5 min at 95°C; followed by 32 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 60 sec; followed by a final extension step at 72°C for 5 min. PCR products were diluted 1:10 with sterile Type I water and centrifuged to mix. PCR products were stored at 4°C for up to 24 h before completion of the next step.

Next, qPCR reactions were conducted using the *M. hapla*-specific qPCR primers Mh-ITSf and Mh-ITSr, which amplify a 136 bp segment within the previously amplified ITS1 product (Hay et al. 2016). Reactions were run in duplicate, and contained 2 µl diluted PCR product as template, 0.15 µM of each forward and reverse primer, 1× SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories), 0.075× SYBR Green-I (Fisher Scientific), and sterile Type I water to bring the volume to 20 µl. Reactions were placed into 96-well white PCR plates (Bio-Rad Laboratories) and sealed with Microseal B plate sealing film (Bio-Rad Laboratories). Thermal cycling reaction

conditions consisted of an initial denaturation of 3 min at 95°C, followed by 50 cycles of 10 sec at 95°C and 30 sec at 60°C with fluorescence readings taken at the end of each combined annealing and extension step. Reactions were run on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories). Reactions were programmed, monitored, standard curves plotted, and unknown quantities analyzed using CFX Maestro software program (Bio-Rad, version 4.1).

Samples for use within a standard curve were generated by inoculating 100 g of sterilized, *M. hapla*-free soil with 100, 500, 1,000, 3,000, and 5,000 *M. hapla* J2s, followed by air drying for 10 days. *M. hapla* J2s were generated using a hydroponics rearing system (Gorny et al. 2018; Appendix A). DNA was extracted using the SPION capture method and nested qPCR assays conducted as described above. Standard curve samples were run in duplicate and included on each reaction plate. Quantities of unknown samples were determined by plotting the resultant quantification cycle on the generated standard curve. Non-inoculated soil subjected to the SPION capture method was used as a non-template control, and DNA of *M. arenaria* (800 pg) was the negative control. DNA of *M. hapla* in pure culture (1,000 pg) was used as a positive control and prepared by plucking 25 *M. hapla* J2s from an inoculum solution extracted using the modified Whitehead tray method described above from a New York State isolate maintained on tomato (cv. Rutgers) in the glasshouse. Nematodes were placed into 500 µl sterile Type I water, and DNA was extracted using the method described by Yan et al. (2008). DNA concentrations of standard curve and control samples were assess using a Qubit fluorometer and stored at -80°C prior to use. A melt curve analysis was included at the end of each reaction run (60 to 95°C, in 0.5°C increments), and a positive reaction exhibited a melting peak at 82°C.

Assay sensitivity and specificity. The sensitivity of the assay was assessed by inoculating 100 g of sterilized, *M. hapla*-free soil with 1, 5, 10, 100, or 1,000 *M. hapla*

J2s. Inoculated soil was air dried for 10 days, then the SPION capture DNA extraction was performed and the nested qPCR assay conducted as described above, with each inoculation level replicated in triplicate. Products from the nested qPCR sensitivity assay were separated on a 1.5% agarose gel stained with 1× GelRed at 70 V for 90 min, and migration of bands compared to a 100 bp ladder (Axygen Scientific, Corning Inc., Corning, NY). The lower limit of detection was defined as the lowest inoculation level that produced a positive real-time amplification signal within the nested qPCR assay and also produced visible bands in the gel electrophoresis.

To assess specificity of the nested qPCR assay to *M. hapla*, DNA from single nematode extracts of 15 non-target plant-parasitic nematodes (*M. incognita, M. fallax*, *M. enterolobii*, *M. javanica*, *M. arenaria*, *M. chitwoodi*, *M. minor*, *M. nassi*, *Pratylenchus penetrans*, *P. neglectus*, *P. thornei*, *Heterodera schachtii*, *H. trifolii*, *H. glycines*, and *Ditylenchus destructor*) and five isolates of *M. hapla* (three from New York State, and one each from The Netherlands and Australia) were tested using the nested qPCR assay as described above. Products from the nested qPCR specificity assay were separated on a 1.5% agarose gel stained with 1× GelRed at 70 V for 90 min, and migration of bands compared to a 100 bp ladder (Axygen Scientific).

Statistical analysis. Data were pooled by year for analysis as no significant differences were found in the mean or variances of the *M. hapla* populations and yield data among the three sampling grids for each year. The associations between *M. hapla* P_i quantified through manual counting and qPCR assays were explored first by visualization of the data through plotting, then assessed through Pearson's correlation coefficients using the function 'cor.test' in R (v. 3.3.3; R Core Team), after $\log(x + 1)$ transformation of populations to fit assumptions of normality. Associations between *M. hapla* P_i (derived from both manual extraction and qPCR estimation), *M. hapla* P_f, total yield, tuber number per meter, average tuber diameter, and average tuber galling

severity were also explored first through visualization of data through plotting, then by Pearson's correlation coefficients. The association between the ratio of *M. hapla* P_f/P_i determined through manual extraction (representing the reproductive factor) and yield in both years of the study was also explored by Pearson's correlation coefficient. When significant, associations were further quantified using simple linear regression analysis using the function 'lm' in R, and the predictive relationship between the variables were described by the equation representing the fitted regression line, and the coefficient of determination (R^2), as a measure of how close the data were to the fitted line.

Results

Detection and quantification of *M. hapla*. *Assay sensitivity and specificity*. The nested qPCR assay had a lower limit of detection of approximately 100 *M. hapla* J2s per 100 cm^3 of soil (Fig. 5.1; Table 5.2). The nested qPCR assay amplified all *M. hapla* isolates tested and none of the non-target species, with the exception of *M. enterolobii*, in which amplification of the same region was noted by a melting curve peak occurring at the same temperature as positive *M. hapla* samples (Fig. 5.2; Table 5.3).

Manual extraction and quantification. M. hapla J2s were found in all grids and fields in both years, and population densities were highly variable within grids in both years. *M. hapla* P_i ranged from 0 to 1,587 J2s per 200 g soil (mean (μ) = 80, standard deviation (SD) = 172) in 2016, and from 0 to 586 J2s per 200 g soil (μ = 104, SD = 106) in 2018. *M. hapla* P_f ranged from 0 to 23,337 J2s per 200 g soil (μ = 1,888, SD = 3,425) in 2016, and from 0 to 3,908 J2s per 200 g soil (μ = 371, SD = 604) in 2018. The average *M. hapla* P_f/P_i was 23.7 in 2016, and 3.6 in 2018.

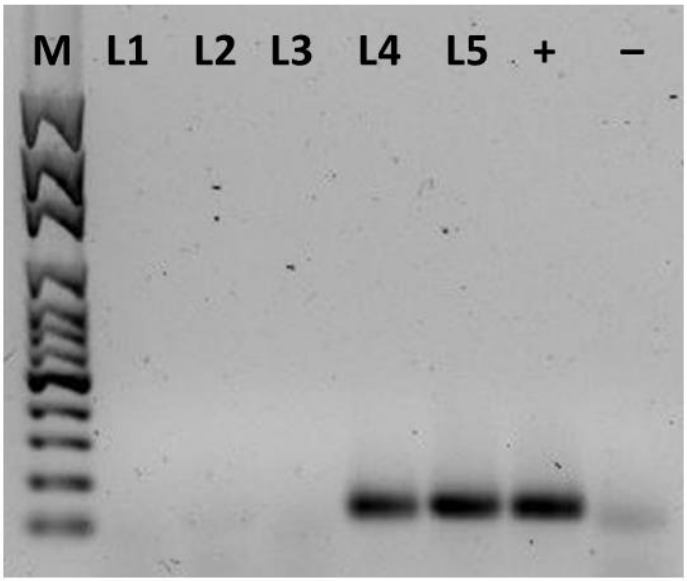

Fig. 5.1. The sensitivity of the nested quantitative PCR reaction was assessed by inoculation of increasing amounts of *Meloidogyne hapla* second-stage juveniles (J2s) to aliquots of 100 g of soil, and performing the SPION capture DNA extraction method and nested qPCR procedure described in the text. M: 100 bp DNA ladder. Lane 1: one *M. hapla* J2. Lane 2: five *M. hapla* J2s. Lane 3: 10 *M. hapla* J2s. Lane 4: 100 *M. hapla* J2s. Lane 5: 1,000 *M. hapla* J2s. Remaining lanes: positive (+) and negative (–) controls.

Table 5.2. A nested quantitative PCR (qPCR) assay targeting the internal transcribed spacer region for detection and quantification of *Meloidogyne hapla* was tested for sensitivity using aliquots of 100 g of soil inoculated with increasing amounts of *M. hapla*, and extracted using the SPION capture DNA extraction method described in the text.

***M. hapla* inoculation density**	**+/- qPCR Signal[a] (Cq)**	**Melting Curve Peak (°C)**
1	-	-
5	-	-
10	-	-
100	+ (16.81)	82.0
1,000	+ (10.49)	82.0

[a] Isolates marked as positive produced a positive signal during quantitative PCR. Isolates marked as negative produced no signal. Corresponding Cq values from each isolate producing a positive signal are included in parentheses.

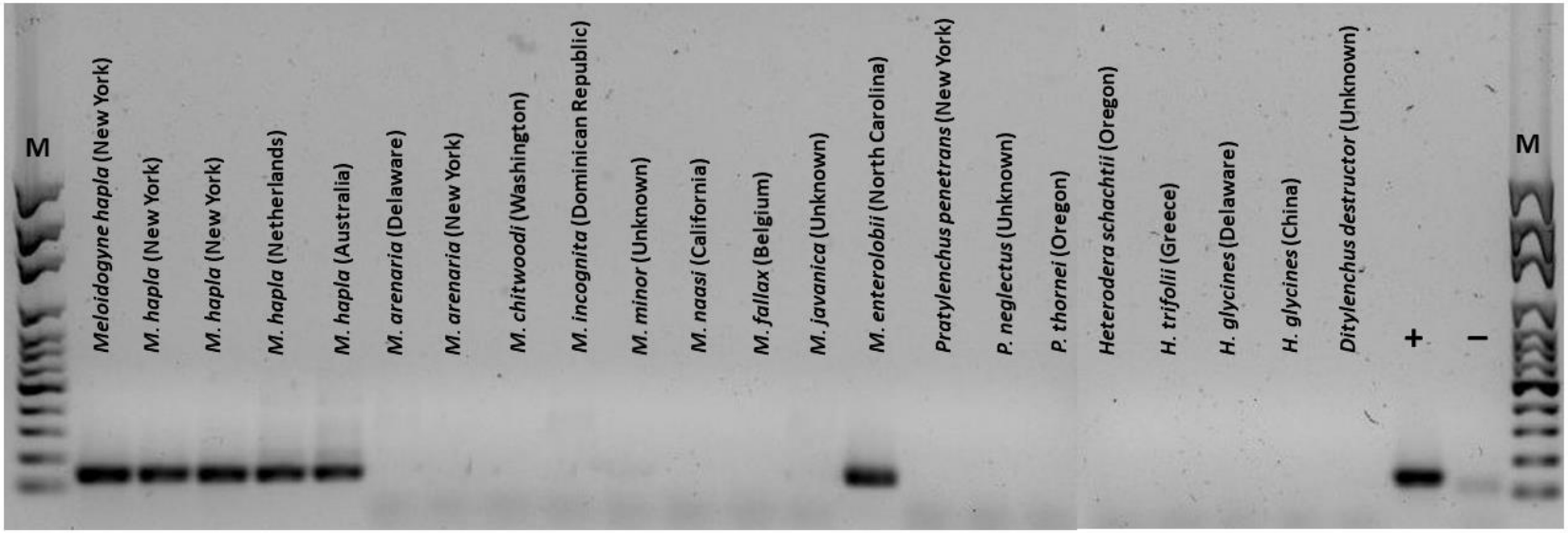

Fig. 5.2. The specificity of the nested quantitative PCR reaction targeting the internal transcribed spacer region for detection and quantification of *Meloidogyne hapla* was assessed by performing the procedure as described in the text on single nematode extracts of 5 isolates of *M. hapla* and 15 non-target plant-parasitic nematode species. Species names and origin of specimen are given above each lane. M: 100 bp DNA ladder. Remaining lanes: positive (+) and negative (–) controls.

Table 5.3. A nested quantitative PCR (qPCR) assay targeting the internal transcribed spacer region for detection and quantification of *Meloidogyne hapla* was tested for specificity against isolates of *M. hapla* and other plant-parasitic nematodes.

Species	Origin	Source	+/- qPCR Signal[a] (Cq)	Melting Curve Peak (°C)
Meloidogyne hapla	New York	S. Pethybridge	+ (6.25)	82.0
M. hapla	New York	S. Pethybridge	+ (18.07)	82.0
M. hapla	New York	S. Pethybridge	+ (13.10)	82.0
M. hapla	Netherlands	A. Skantar	+ (13.04)	82.0
M. hapla	Australia	N. Perry	+ (10.42)	82.0
M. arenaria	Delaware	A. Skantar	-	-
M. arenaria	New York	L. Cadle-Davidson	-	-
M. chitwoodi	Washington	A. Skantar	-	-
M. incognita	Dominican Republic	A. Skantar	-	-
M. minor	Unknown	A. Skantar	-	-
M. naasi	California	A. Skantar	-	-
M. fallax	Belgium	A. Skantar	-	-
M. javanica	Unknown	N. Perry	-	-
M. enterolobii	North Carolina	E. Davis	+ (23.88)	82.0
Pratylenchus penetrans	New York	S. Pethybridge	-	-
P. neglectus	Unknown	A. Skantar	-	-
P. thornei	Oregon	A. Skantar	-	-
Heterodera schachtii	Oregon	A. Skantar	-	-
H. trifolii	Greece	A. Skantar	-	-
H. glycines	Delaware	A. Skantar	-	-
H. glycines	China	A. Skantar	-	-
Ditylenchus destructor	Unknown	A. Skantar	-	-

[a] Isolates marked as positive produced a positive signal during quantitative PCR. Isolates marked as negative produced no signal. Corresponding Cq values from each isolate producing a positive signal are included in parentheses.

qPCR estimation. Quantification of *M. hapla* P_i through the nested qPCR assay also indicated populations were highly variable. Estimates of *M. hapla* P_i were generally higher using the qPCR assay, ranging from 0 to 255,000 *M. hapla* J2s per 200 g soil (µ = 10,228, SD = 35,150) in 2016, and from 0 to 61,900 *M. hapla* J2s per 200 g soil (µ =1,927, SD = 4,942) in 2018. There was a significant positive association between *M. hapla* P_i quantified through manual extraction and the nested qPCR assay in 2016 and 2018 (r = 0.47 and 0.33, $P < 0.0001$; Table 5.4), and the combined data was described by the linear regression equation: $y = 1.03x - 0.018$ with low predictive power ($R^2 = 0.175$, $P < 0.0001$; Fig. 5.3). *M. hapla* P_i determined through manual extraction was significantly correlated with P_f in both years (r = 0.46 and 0.295, $P < 0.0001$; Table 5.4), and the combined predictive relationship was described by the equation: $y = 0.61x + 1.1$ ($R^2 = 0.14$, $P < 0.0001$). *M. hapla* P_i estimated through the nested qPCR assay was also significantly correlated with P_f in both years (r = 0.469 and 0.157, $P < 0.01$; Table 5.4) and the combined predictive relationship could be described by the equation: $y = 0.23x + 1.71$ ($R^2 = 0.11$, $P < 0.0001$).

Relationship between *M. hapla* populations and potato yield components. In 2016 and 2018, *M. hapla* P_i quantified by manual counting were not significantly associated with total yield (r = 0.076 and 0.105, $P > 0.05$), number of tubers (r = 0.024 and -0.01, $P > 0.05$), tuber diameter (r = 0.01 and 0.029, $P > 0.05$), or tuber galling severity (r = -0.01 and 0.028, $P > 0.05$; Table 5.4). Overall, tuber galling severity scores were very low in both years, with an average of 0.88 (range of 0 to 3) and 0.29 (range of 0 to 2), respectively. In 2016 and 2018, *M. hapla* P_i estimated through the nested qPCR assay was also not significantly correlated with total yield (r = 0.11 and 0.111, $P > 0.05$), number of tubers (r = 0.096 and 0.096, $P > 0.05$), tuber diameter (r = -0.002 and -0.005; $P > 0.05$), or tuber galling severity (r = -0.052 and 0.066, $P > 0.05$; Table 5.4). *M. hapla* P_f was significantly positively correlated with total yield in 2018 (r = 0.177, $P = 0.002$;

Table 5.4) and described by the equation: $y = 0.119x + 2.544$ ($R^2 = 0.031$, $P = 0.002$). However, there was no correlation between *M. hapla* P_f and yield in 2016 (Table 5.4). *M. hapla* P_f in 2016 was significantly positively correlated with number of tubers (r = 0.293, $P < 0.0001$) and described by the equation: $y = 2.5x + 23.92$ ($R^2 = 0.09$, $P < 0.0001$). *M. hapla* P_f in 2016 was significantly negatively correlated with tuber diameter (r = - 0.147, $P < 0.0001$), and described by the equation: $y = -1.64x + 64.9$ ($R^2 = 0.02$, $P < 0.0001$). *M. hapla* P_f in 2016 was also significantly negatively correlated with tuber galling severity (r = - 0.136, $P = 0.018$; Table 5.4), and described by the equation: $y = -0.08x + 0.936$ ($R^2 = 0.02$, $P = 0.018$). The ratio of *M. hapla* P_f/P_i determined through manual extraction was not significantly correlated with yield in 2016 (r = 0.026, $P > 0.05$). However, the relationship between *M. hapla* P_f/P_i was significantly positively associated with yield in 2018 (r = 0.134, $P = 0.02$) and was described by the equation: $y = 0.14x + 2.63$ ($R^2 = 0.02$, $P = 0.02$).

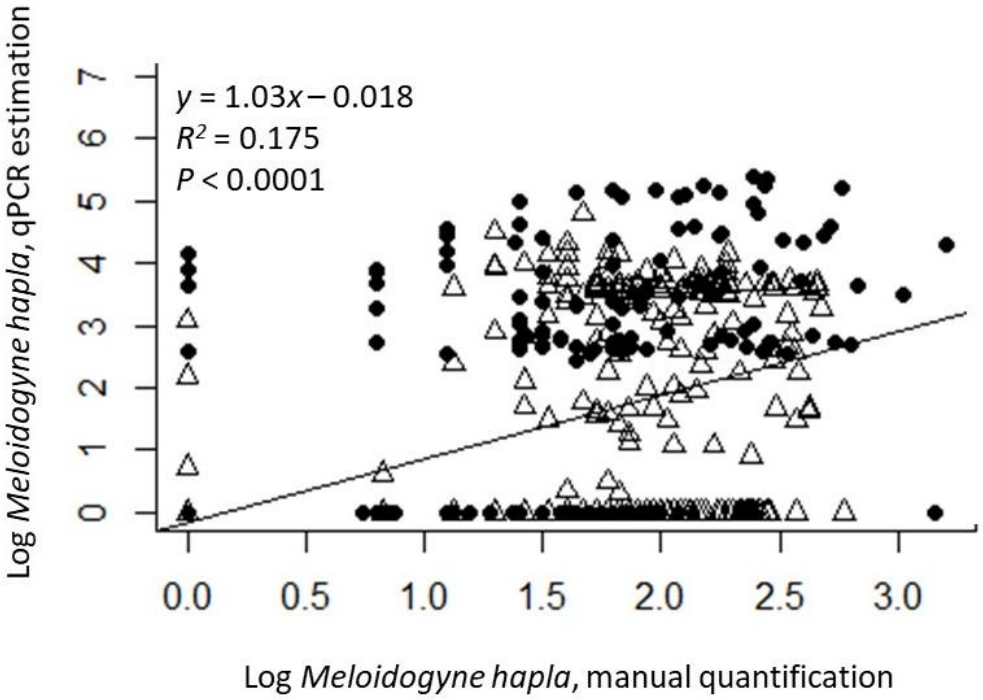

Fig. 5.3. Plot of initial *Meloidogyne hapla* populations determined through manual quantification and a nested qPCR assay, plotted on a log scale evaluated from soil samples collected in commercial potato fields in New York State in 2016 (filled circle) and 2018 (open triangle).

Table 5.4. Pearson's correlation coefficient matrix among initial (P_i) and final (P_f) *Meloidogyne hapla* populations determined though manual extraction and a nested quantitative PCR assay, and potato yield components, including total yield, number of tubers, tuber diameters, and tuber galling severity in New York State in 2016 and 2018. $P < 0.0001$ (***), $P < 0.001$ (**), $P < 0.05$ (*), $P < 0.1$ (·). *M. hapla* populations quantified by manual extraction and qPCR assay were log(x +1) transformed prior to analysis.

	Manual P_i[a]	**Manual P_f[a]**	**SPION P_i[b]**	**Total Yield[c]**	**No. Tubers[d]**	**Diameters[e]**	**Galling Severity[f]**
				2016			
Manual P_i	1.0						
Manual P_f	0.460 ***	1.0					
SPION P_i	0.473 ***	0.469 ***	1.0				
Total Yield	0.076	0.022	0.110 ·	1.0			
No. Tubers	0.024	0.293 ***	0.096	0.571 ***	1.0		
Diameter	0.010	- 0.147 ***	- 0.002	0.243 ***	- 0.204 ***	1.0	
Galling Severity	- 0.010	- 0.136 *	- 0.052	0.297 ***	0.101 ·	0.147 ***	1.0
				2018			
Manual P_i	1.0						
Manual P_f	0.295 ***	1.0					
SPION P_i	0.333 ***	0.157 *	1.0				
Total Yield	0.105 ·	0.177 *	0.111 ·	1.0			
No. Tubers	- 0.010	0.048	0.057	0.766 ***	1.0		
Diameter	0.029 ·	0.010	- 0.005	0.036 *	- 0.104 ***	1.0	
Galling Severity	0.028	- 0.003	0.066	0.072	0.073	0.004	1.0

[a] Initial (P_i) and final (P_f) *M. hapla* populations determined through manual extraction and quantification.

[b] Initial *M. hapla* populations estimated through DNA isolation by super paramagenetic iron oxide nanoparticle (SPION) extraction followed by population estimation by nested quantitative PCR.

[c] Total tuber yield per sampling location (kg).

[d] Number of tubers per sampling location.

[e] Average tuber diameter (mm; measured on the long axis).

[f] Tuber galling severity (scored on a scale of 0 to 6; scale adapted from Baldwin et al. 2008).

Discussion

M. hapla P_i estimated through the nested qPCR assay were significantly positively correlated with those quantified through manual extraction, indicating that the nested qPCR assay was robust in determining the relative population densities of *M. hapla* in soil. Minor cross reactivity was observed in the nested qPCR assay with *M. enterolobii*, with a lesser amount of amplification occurring, but amplicons exhibiting the same melt peak as *M. hapla*-positive samples. *M. enterolobii* is not present in New York State (Anonymous 2014), however, care should be taken if the qPCR assay were used in regions where both species are present.

A high proportion (48%) of samples identified as containing *M. hapla* through manual extraction produced negative signals in the nested qPCR assay. This may potentially have been due to the influence of soil inhibitors on the amplification reactions (Sidstedt et al. 2015). Alternatively, this discrepancy may also have been due to true population densities below the detection threshold of the nested qPCR assay of approximately 100 *M. hapla* J2s per 100 g of soil. Other studies have also described discrepancies in the estimation of nematode populations from soil using different techniques. For example, Stirling et al. (2004) found that a DNA-based molecular assay for *Meloidogyne* spp. produced similar results to standard extraction techniques and neither was accurate for predicting low populations. Based upon these results, this nested qPCR assay could be used in place of manual extraction and quantification for estimation of *M. hapla* prior to planting potato despite the lower limit of detection (approximately 100 *M. hapla* J2s per 100 g soil), as it does not appear populations below this level were associated with yield or quality losses. However, the assay would have limited utility as a pre-plant test for soil testing for highly sensitive crops (i.e. carrot and onion) (Melakeberhan et al. 2012).

No significant associations were observed between *M. hapla* P_i quantified through either manual extraction or the nested qPCR assay, and selected potato yield components (total yield, number of tubers, average tuber diameter, or average tuber galling severity), suggesting that population densities quantified in this study were not a significant determinant of potato yield or tuber quality. However, the average P_f/P_i ratios of 23.7 and 3.6 in 2016 and 2018, respectively, indicate that populations were increasing on potato during the season, and the cultivars are a suitable host of *M. hapla* but likely tolerant.

In 2016, high variability in population density and total yield (coefficient of variation = 215% and 44%, respectively) was observed among sampling points. This variability suggested that other spatially dependent biotic and abiotic factors were likely influencing populations and total yield. Therefore, in order to reduce the effect of extraneous factors, the distance between grid points was reduced in 2018. This resulted in a narrower range of values for *M. hapla* population densities and total yield (coefficient of variation = 163% and 23%, respectively). Yet even with this adjustment, *M. hapla* P_i was not significantly associated with yield in either year irrespective of quantification method.

In order to calculate the economic threshold for a pathogen in a crop, the corresponding yield loss must be quantified (Fiers et al. 2012). Approximately 25% of samples collected at the initial sampling had populations at or above the previously defined economic threshold of 100 *M. hapla* J2s per 100 g soil. However, no significant reductions in yield were observed with increasing *M. hapla* P_i, thus an appropriate economic threshold for *M. hapla* in potato production could not be calculated. The economic threshold for *M. hapla* in potato was previously 100 J2s per 100 g of soil (Olthof and Potter 1972; Barker and Olthof 1976), yet these findings do not support this and potentially suggest that the economic threshold for *M. hapla* in New York State

potato production may be higher than the initial population densities observed in the sampled fields.

Reports of yield reductions in the *M. hapla*-potato pathosystem are mixed and range from substantial (Griffin and Jorgenson 1969; Olthof and Potter 1972; Brodie et al. 1993) to mild, and less than calculated from other *Meloidogyne* spp. (Melakeberhan et al. 2012). Effects on yield and resultant damage may also be strongly dependent upon edaphic and environmental factors including soil temperature and texture (Griffin and Jorgenson 1969; Wong and Mai 1973; Griffin 1979; Santo and O'Bannon 1981). Griffin (1979) noted that soil temperature had a significant influence on tuber galling, with no galling occurring when soil temperatures were below 20°C. Mean soil temperature at 20 cm (representing the root-zone) was above 20°C for 72 days in 2016, and 48 days in 2018 (data retrieved from the U.S. Department of Agriculture – National Water and Climate Center; Station #2011 Geneva, NY). The average generation time for many *Meloidogyne* spp. is 28 to 35 days at optimal base temperatures of approximately 24 to 28°C (Bridge and Starr 2007). These conditions only therefore represent sufficient time for approximately two generations in 2016 and one generation in 2018, likely reflected in the low average tuber galling severity scores observed. Griffin and Jorgenson (1969) noted that growth of potato plants was affected by both air temperature and *M. hapla* isolate pathogenicity. Further, Kandouh and Sipes (2014) noted that some red-skinned potato cultivars were susceptible, but tolerant to infection by different *Meloidogyne* spp., noting no significant difference in tuber weight between infected and non-infected plants. Soil texture has also been correlated with nematode movement in soil, with coarse-textured (low clay content) soils facilitating increased migration of *Meloidogyne* spp. and increased crop damage (Prot and Van Gundy 1981; Shane and Barker 1986; Koenning 1996). The fields sampled in this study were a sandy loam to silty loam texture, with clay contents ranging between 9.9 to 14.1%, which may have inhibited

migration of *M. hapla* through the soil profile. Economic thresholds for *M. hapla* and other plant-parasitic nematode species may therefore be different depending upon the soil texture in which the crop is grown.

Other studies have also observed differences in pathogenicity among *M. hapla* isolates to potato and other crops (Van der Beek et al. 1998; Roberts and Chen 2003; Liu and Williamson 2006). For example, Mitkowski and Abawi (2003) observed differing levels of pathogenicity (measured as reproductive fitness and severity of root galling) among six different isolates of *M. hapla* from New York State on lettuce, and recommended that germplasm susceptibility evaluations include isolates representative of multiple populations to account for this variation. These results support the finding that the potato cultivars included in this study may be susceptible, but tolerant to *M. hapla*, or that isolates of *M. hapla* encountered during the study may have reduced pathogenicity on potato. Collecting data on the virulence and aggressiveness of the *M. hapla* isolate present in commercial potato fields (in addition to quantifying initial populations) would underpin the development of additional, locally relevant yield loss models in potato and other pathosystems.

The economic implications of the finding that 100 *M. hapla* J2s per 100 g of soil is inappropriate as an economic threshold for these potato cultivars in New York State for producers are substantial. A pre-plant nematicide is a considerable variable cost to potato production (e.g., Vydate® C-LV (DuPont™, Midland, MI) estimated at approximately $148 USD/ha, not including application expenses; K. Hofmann, personal communication). Assuming that *M. hapla* is the most significant pest target for this application, withdrawal would have economic consequences to the profit margin of the crop, and additional environmental benefits (e.g., Baird et al. 2001; King and Taberna 2013). Further research evaluating the variability between *M. hapla* populations and their pathogenicity to these cultivars, the role of soil temperature and moisture as a yield

determinant, and interactions with other plant-parasitic nematodes may improve our knowledge of yield loss relationships in New York State potato production and underpin improved decision making for soilborne disease control.

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CHAPTER 6

RESPONSES OF TWO POTATO CULTIVARS TO THE NORTHERN ROOT-KNOT NEMATODE, *MELOIDOGYNE HAPLA*, AND ROOT-LESION NEMATODE, *PRATYLENCHUS PENETRANS*, IN NEW YORK STATE

Abstract

The Northern root-knot nematode (RKN), *Meloidogyne hapla*, and the root-lesion nematode, *Pratylenchus penetrans*, are two important soilborne pathogens of potato. Differing levels of susceptibility in potato to these nematodes have been described, yet the response of newer cultivars has not been studied. Here, the popular potato cvs. Eva and Lamoka were assessed for their response to *M. hapla* and *P. penetrans* through replicated field trials. Potato tubers were inoculated with variable initial population densities of each species, and yield, tuber set and diameters, and galling severity was assessed at harvest. Final population densities were estimated through nematode extraction from soil samples collected at harvest. Initial population density of either species did not have a significant effect on tuber yield, but *M. hapla* populations had a significant effect on tuber set and diameter in one of the two trials. This study provides insights into the response and host status of the potato cvs. Eva and Lamoka to *M. hapla* and *P. penetrans* populations. The tolerance of these cultivars may facilitate preferential selection in fields where *M. hapla* and *P. penetrans* populations are high and the absence of a trade-off in yield or quality may negate the need for pre-plant nematicide usage.

Introduction

Potatoes are an important vegetable crop in New York State. In 2017, approximately 5,830 hectares of potatoes were harvested equating to approximately $50.8 million USD, placing New York State thirteenth nationally in potato production (Anonymous 2017). Potatoes produced in New York State include those for fresh and processing (chipping) markets, with a broad diversity of cultivars including white, red, and yellow tablestock potatoes, and specialty types such as fingerlings and blue skinned potatoes (L. Stivers, N.D., Cornell CE).

In New York State and the Northeastern United States, the Northern root-knot nematode (RKN), *Meloidogyne hapla*, and the root-lesion nematode, *Pratylenchus penetrans*, are frequently observed in potato fields (Brodie and Plaisted 1993; Mitkowski et al. 2002). The second-stage juveniles (J2s) of *M. hapla* (Fig. 6.1A) invade fine root hairs immediately behind the root cap, and migrate intercellularly before establishing a fixed feeding site within the vascular tissues (Perry et al. 2009; Jones et al. 2013). As the nematode matures into an adult, females swell in size (Fig. 6.1B), leading to the characteristic root galls (Fig. 6.1C), which may inhibit root function and deleteriously impact plant growth (Perry et al. 2009). *M. hapla* may also invade tubers directly, causing galling and knotting on the surface (Fig. 6.1D).

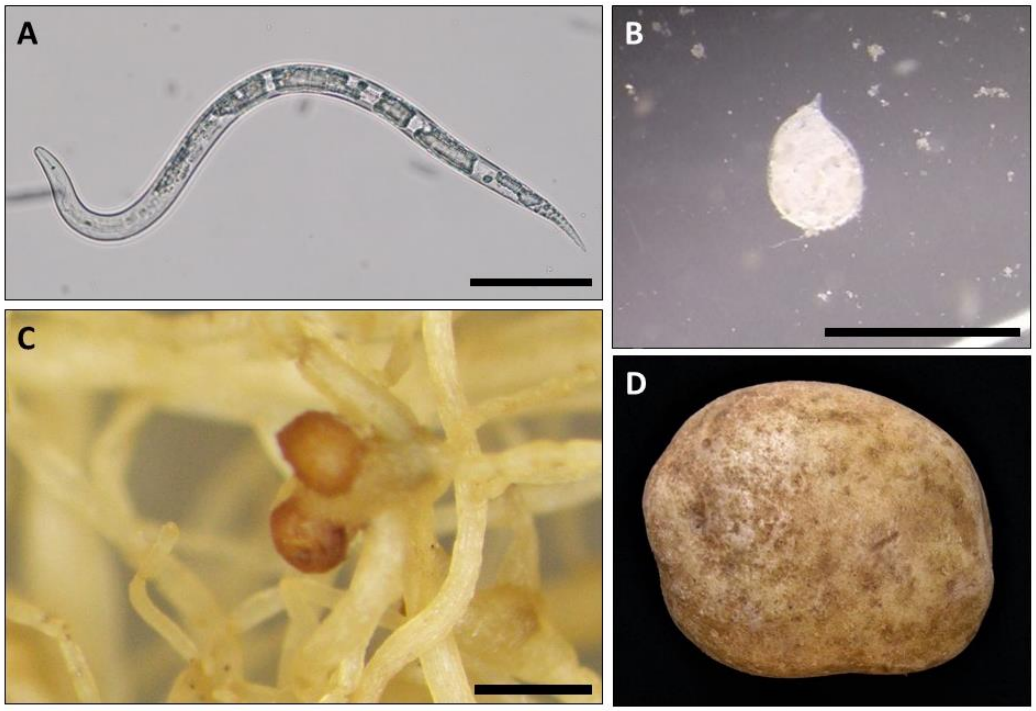

Fig. 6.1. Life stages of the Northern root-knot nematode (*Meloidogyne hapla*): Second-stage juvenile (**A**; scale = 50 µm), adult female (**B**; scale = 500 µm), root gall and mature egg sack of *M. hapla* on potato roots (**C**; scale = 500 µm), and galling damage on a potato cv. Eva tuber (**D**).

The root-lesion nematode, *P. penetrans* (Fig. 6.2A) has a migratory lifestyle and juvenile stages and adult nematodes may invade host roots to feed on cortical, epidermal, and endodermal tissues (Jones et al. 2013). Feeding therefore results in necrotic lesions, dieback and reduction in root volume (Bridge and Starr 2007; Jones et al. 2013). *P. penetrans* may also invade tubers directly, and damage is similar to common scab lesions caused by *Streptomyces* spp. (Fig. 6.2B; Holgado et al. 2009).

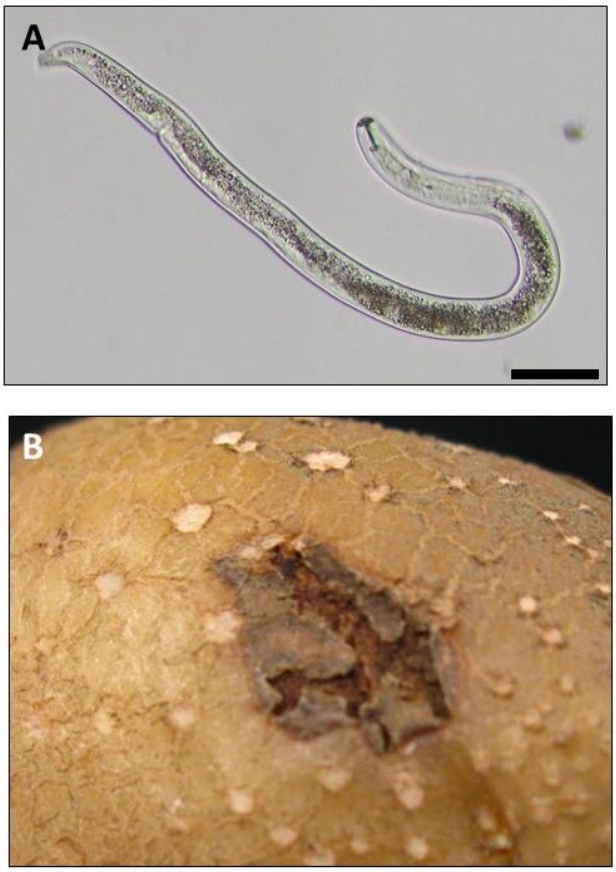

Fig. 6.2. Adult female root-lesion nematode (*Pratylenchus penetrans*) (**A**; scale = 50 µm) and damage caused by *P. penetrans* to a potato tuber, cv. Eva (**B**).

Tuber damage by *M. hapla* or *P. penetrans* may cause rejection for fresh market sales or requiring additional peeling to remove blemished tissue for processing. In addition to quality losses, *M. hapla* and *P. penetrans* may also reduce yield (Dickerson et al. 1964; Olthof and Potter 1972; MacGuidwin and Rouse 1990; Philis 1995; Holgado et al. 2009). For example, Olthof and Potter (1972) found yield for the potato cv. Sebago decreased and the number of culls increased when *M. hapla* populations ranged from 166 to 4,500 J2s per 250 g soil. Bernard and Laughlin (1976) observed yield losses of 20 to 30% on cv. Superior, even at low densities of 38 *P. penetrans* nematodes per 100 g soil.

No commercially available potato cultivars possess genetic resistance to *M. hapla* (Brown et al. 1994; Janssen et al. 1995; Janssen et al. 1996; Van de Beek et al. 1998; Melakeberhan et al. 2012). Some studies (Van der Beek et al. 1998; Melakeberhan et al. 2012; Kandouh and Sipes 2014) have noted differences in susceptibility to *Meloidogyne* spp. between cultivars, reflected in yield, plant vigor, and nematode reproductive measures. In a greenhouse study, Melakeberhan et al. (2012) observed different final populations of *M. hapla* on diverse potato cultivars, suggesting differences in host status. Likewise, there is a lack of genetic resistance to *P. penetrans* in commercial potato cultivars (Brodie and Plaisted 1993), yet differential susceptibility to *P. penetrans* among potato cultivars has also been reported (Olthof and Potter 1973; Bernard and Laughlin 1976; Brodie and Plaisted 1993; France and Brodie 1995). Bernard and Laughlin (1976) observed differential tolerance by quantifying yield reductions in the cvs. Katahdin, Kennebec, and Superior when experimental microplots were inoculated with moderate populations (approximately 80 *P. penetrans* J2s per 100 cm^3 of soil), but no significant yield reduction was observed at similar populations in the cv. Russet Burbank. Some research evaluating the susceptibility of cv. Hudson to *P. penetrans* first reported it as a poor host, supporting relatively lower populations at harvest than other cultivars tested (Dunn 1973; Fawole and Mai 1988). Yet other studies found cv. Hudson to be good host, with populations at harvest significantly higher in plots planted with Hudson than other cultivars tested (Kotcon et al. 1987; France and Brodie 1995). *Pratylenchus* species, including *P. penetrans*, are also part of the early dying disease complex in association with several soilborne fungal pathogens, most commonly, *Verticillium dahliae* (Powelson and Rowe 1993). The presence of *P. penetrans* and *V. dahliae* may lead to greater yield loss when compared to losses due to either pathogen alone (Martin et al. 1982; Rowe et al. 1985).

Crop yield and damage associated with plant-parasitic nematodes is often correlated with initial population density (Seinhorst 1965) and several studies have aimed to identify the economic threshold for *M. hapla* and *P. penetrans* in potato. Stern et al. (1959) defined the economic threshold as the pest population at which control measures should be implemented, in order to hinder the population from increasing, resulting in yield loss or crop damage that justifies the cost of control. Earlier research using the cv. Sebago suggested an economic thresholds of 100 *M. hapla* J2s per 100 g of soil (Barker and Olthof 1976; Olthof and Potter 1972), and 200 to 600 *P. penetrans* per 100 g soil (Barker and Olthof 1976; Olthof and Potter 1973). Holgado et al. (2009) also suggested an economic threshold of 100 *P. penetrans* per 250 g soil for cv. Saturna. Little is known of the susceptibility of more recently introduced and popular potato cultivars to *M. hapla* and *P. penetrans.* Given the prevalence of high populations in New York State cropping rotations, their broad host ranges limiting the ability to use crop rotation for management, and the risk averse nature of growers necessitating prophylactic pre-plant nematicide application, additional information on cultivar susceptibility would facilitate field-specific management decisions.

The objective of this study was to quantify yield loss, crop damage, and relative host status of two more recently released potato cvs. Lamoka and Eva to *M. hapla* and *P. penetrans* under field conditions. These cultivars were selected due to their popularity among New York State potato growers and Northeastern consumers. Lamoka is a white skinned chipping potato that has resistance to the golden potato cyst nematode (*Globodera rostochiensis*) R01 race (De Jong et al. 2017). Eva is a white skinned tablestock potato also possessing resistance to the R01 race of *G. rostochiensis* (Plaisted et al. 2001). Seed pieces of both cultivars were provided by the Cornell potato breeding program (Cornell University, Ithaca, NY). It was hypothesized that higher initial population densities of *M. hapla* or *P. penetrans* would result in reduced yield and

increased damage in both cultivars. Further, it was hypothesized that higher initial population densities would result in higher final populations of both species, in both cultivars.

Materials and Methods

Trial Design. Field trials were conducted in each of two years (2017 and 2018) at the H. C. Thompson Vegetable Research Farm in Freeville, NY (N 42°51'; W -76°33'). The soil type was a gravelly loam soil with low initial population densities of *M. hapla* and *P. penetrans* (≤ 10 *M. hapla* or *P. penetrans* idividuals per 200 g soil). The trial design was a split plot with two blocks, each separately evaluating the effect of *P. penetrans* and *M. hapla*. Each block was 17.6 m long and 21 rows wide, with 0.86 m between row spacing. Blocks contained two treatment factors, including potato cultivar (Eva or Lamoka) and initial nematode population (P_i). Treatment plots were 2 m long and contained five potato plants in a single row at a spacing of 0.3 m. Treatments were replicated over six plots. Guard rows of red skinned potatoes were planted between plot across rows, and two guard plants between plots within rows.

Prior to trial establishment, potato seed pieces were planted into 10.2 cm diameter biodegradable peat pots (Griffin Greenhouse and Nursery Supply, Tewksbury, MA) and filled with a sterilized top-soil mix (three parts top-soil to one part sand). Seed pieces were maintained in the greenhouse (25°C, 14 h photoperiod), and were inoculated three weeks after emergence plants by making three small holes (approximately 1 cm wide × 5 cm deep) around the base of each plant and delivering an appropriate amount of inoculum solution through uniformly watering with a pipette. Sterilized water of the same volume was delivered to the non-inoculated controls in the same manner. *M. hapla* inoculum solution was prepared from a hydroponics rearing unit (Gorny et al. 2018; Appendix A). Briefly, hydroponic reservoir water containing *M. hapla* J2s was siphoned

off and passed through a 25 micron mesh sieve held at a 45° angle. Nematodes were collected from the sieve and quantified under 100× magnification and the concentration of nematodes per ml was calculated. *M. hapla* nematodes were inoculated at initial populations of 250 (medium P_i) and 500 (high P_i) per plant. A non-inoculated control was also included. *M. hapla* J2s were retrieved from the hydroponics, populations quantified, and used to inoculate plants on the same day to reduce the probability of nematode attrition.

P. penetrans inoculum was obtained by rearing a single-species colony on hairy vetch (*Vicia villosa*) plants in the greenhouse. Briefly, hairy vetch was seeded into soil infested with *P. penetrans* in 15.2 cm diameter clay pots and maintained for approximately 3 months in the greenhouse (25°C, 14 h light). Soil and root matter were then removed from the pots and live nematodes extracted using a modified Whitehead tray method (Whitehead and Hemming 1965). Water from the extraction trays was passed through a 25 micron mesh sieve held at 45°. Nematodes were washed from the sieve, *P. penetrans* quantified under 100× magnification and the concentration of nematodes per milliliter was calculated. *P. penetrans* were inoculated at initial population densities of 500 (medium P_i) and 1,000 (high P_i) nematodes per plant. A non-inoculated control was also included in the block. *P. penetrans* extraction, quantification, and inoculation was conducted on the same day.

Plants were maintained in the greenhouse for 10 days post inoculation (25°C, 14 h photoperiod), after which they were transplanted into the field by hand (25 May 2017; 22 May 2018). Blocks were maintained with standard agronomic practices for potato, including regular fungicide application for foliar disease control, herbicides for weed management, and supplemental irrigation through overhead sprinklers for optimal plant growth as needed. Plants were chemically vine killed with diquat dibromide (Reglone® at 2.3 l/ha, Syngenta Corporation, Greensboro, NC) at 97 and 91 days after planting

(DAP) in 2017 and 2018, respectively. Tubers were harvested using a tractor-mounted single-row digger at 137 and 133 DAP, in 2017 and 2018.

Data Collection. Potato yield components measured on a plot basis were yield (kg), tuber set (number of tubers formed per plot), and tuber size (diameter across the widest axis; mm). Crop damage was also assessed by evaluating all tubers within a plot as a whole. The powdery scab severity scale of Baldwin et al. (2008) was adapted for use in scoring *M. hapla* damage by exchanging scab severity for root-knot galls on the tuber, from 0 (no galling) to 6 (galls covering tubers). The disease severity scale of Holgado et al. (2009) was used to score *P. penetrans* damage on an ordinal scale, from 0 (no lesions) to 9 (lesions covering tubers). As damage caused by *P. penetrans* looks similar common scab (Holgado et al. 2009), all necrotic lesions of this type were included when assigning severity ratings. The final population density (P_f) of each plot was estimated by taking a 200 g soil sample from the root zone during harvesting. Nematode extraction from soil was performed using a modified Whitehead tray method (Whitehead and Hemming 1965) and populations quantified at 100× magnification.

Statistical Analyses. The main effects of cultivar and P_i, and their interaction on yield, tuber set, tuber size, and P_f per plot were explored for each nematode species using two-way analysis of variance within R (v. 3.3.3; R Core Team 2017), and replication as a random effect. Where significant differences were observed, Tukey's honest significant difference test was used to separate means (function 'HSD.test' within the 'agricolae' package). The effects of P_i on disease severity were assessed using Spearman's rank correlation (function 'cor.test' within the 'stats' package). The assumptions of normality and homogeneity between variances in the data were verified by visualization of the data and the Shapiro-Wilks (function 'shapiro.test') and Levene's tests (function 'leveneTest' within the 'car' package). Data from each year were analyzed separately.

Results

Meloidogyne hapla. No significant main or interactive effects of cultivar or P_i on tuber yield were observed within the *M. hapla* inoculated block, in either year of the study (Table 6.1). Tuber set was significantly influenced by the main effect of cultivar in 2017, with Eva producing an average of 30.6% more tubers than Lamoka (mean (μ) = 25.2; Table 6.2). Tuber set was also significantly influenced by the main effect of P_i in 2017, with the medium P_i (250 nematodes per plant) and the high P_i (500 nematodes per plant) producing an average of 19.1% and 13.0% fewer tubers than the non-inoculated control (μ = 32.4 tubers). However, there was no significant interaction between cultivar and P_i on tuber set in either year (Table 6.2). Tuber diameter was significantly influenced by the main effect of cultivar in both years of the study, with Lamoka tubers 6% and 3.2% larger than Eva (μ = 77.8 and 76.8 mm) in 2017 and 2018, respectively. Tuber diameter was significantly influenced by the main effect of P_i in 2017, with tubers from high P_i plots 2.4% smaller than non-inoculated controls (μ = 78.8 mm), yet no effect was observed in 2018. Tuber diameter was significantly influenced by the interaction of the two factors within the *M. hapla* inoculated block in 2018, but not in 2017 (Table 6.3).

Table 6.1. Effect of selected initial populations of *Meloidogyne hapla* and *Pratylenchus penetrans* on tuber yield (kg) in potato cvs. Eva and Lamoka in small plot replicated trials conducted in 2017 and 2018 at Freeville, New York State. Means (n = 6) followed by the same letter within each nematode species × year grouping are not significantly different at the 0.05 level for interaction of cultivar and initial population density (P_i).

	Year	**2017**		**2018**	
	Cultivar	**Eva**	**Lamoka**	**Eva**	**Lamoka**
Species	**P_i, per pot**				
Meloidogyne hapla	Control (Non-inoculated)	6.67	6.42	4.31	4.65
	Medium (250)	6.57	5.82	4.31	4.29
	High (500)	5.69	5.42	4.23	4.24
	F value =	0.276		0.407	
	P =	0.760		0.671	
Pratylenchus penetrans	Control (Non-inoculated)	5.87	5.92	4.93	5.04
	Medium (500)	5.95	5.88	4.68	4.48
	High (1,000)	6.02	5.72	4.13	4.56
	F value =	0.18		0.734	
	P =	0.880		0.491	

Table 6.2. Effect of selected initial populations of *Meloidogyne hapla* and *Pratylenchus penetrans* on potato tuber set (number of tubers per plot) in the cvs. Eva and Lamoka in small plot replicated trials conducted in 2017 and 2018 at Freeville, New York State. Means (n = 6) followed by the same letter within each nematode species × year grouping are not significantly different at the 0.05 level for interaction of cultivar and initial population density (P_i).

	Year	**2017**		**2018**	
	Cultivar	**Eva**	**Lamoka**	**Eva**	**Lamoka**
Species	**P_i, per pot**				
Meloidogyne hapla	Control (Non-inoculated)	35.2	29.7	31.0	24.6
	Medium (250)	30.6	21.8	25.4	24.8
	High (500)	32.7	23.7	24.6	23.6
	F value =	0.502		2.25	
	P =	0.611		0.128	
Pratylenchus penetrans	Control (Non-inoculated)	30.5	27.5	26.8	25.2
	Medium (500)	29.2	25.8	27.0	23.0
	High (1,000)	31.0	27.5	24.6	21.8
	F value =	0.01		0.257	
	P =	0.990		0.776	

Table 6.3. Effect of selected initial populations of *Meloidogyne hapla* and *Pratylenchus penetrans* on tuber diameter (mm; measured across the wide axis of the tuber) in the cvs. Eva and Lamoka in small plot replicated trials conducted in 2017 and 2018 at Freeville, New York State. Means (n = 6) followed by the same letter within each nematode species × year grouping are not significantly different at the 0.05 level for interaction of cultivar and initial population density (P_i).

	Year	**2017**		**2018**	
	Cultivar	**Eva**	**Lamoka**	**Eva**	**Lamoka**
Species	**P_i, per pot**				
Meloidogyne hapla	Control (Non-inoculated)	79.4	82.1	74.4 b	80.7 a
	Medium (250)	78.1	84.1	78.0 ab	79.1 ab
	High (500)	76.1	82.3	78.4 ab	78.4 ab
	F value =	2.71		3.15	
	P =	0.067		0.043	
Pratylenchus penetrans	Control (Non-inoculated)	78.1	78.8	79.8	80.4
	Medium (500)	80.4	84.1	78.4	77.0
	High (1,000)	78.0	81.0	79.0	82.4
	F value =	0.944		1.51	
	P =	0.390		0.22	

Final nematode population density was significantly influenced by cultivar in both years of the study, with an average of 360% and 155% fewer *M. hapla* nematodes recovered from Lamoka than Eva (μ = 143.6 and 27.8 nematodes) in 2017 and 2018, respectively. Final population was also significantly influenced by the main effect of P_i in both years. In 2017, medium and high P_i plots produced an average of 951% and 2,517% more *M. hapla* than the non-inoculated control (μ = 6.9 nematodes). In 2018, medium P_i and high P_i plots produced an average of 456% and 474% more *M. hapla* than the non-inoculated control (μ = 5.0 nematodes). Final populations were significantly influenced by the interaction of cultivar and P_i in 2017, but not in 2018 (Table 6.4). *M. hapla* populations were recovered from soil samples in all inoculated plots in both years of the study.

Low RKN damage scores (≤ 1.2) were observed on tubers in inoculated plots for both cultivars, equating to approximately 2 to 3% of surface area covered by galls. The Spearman's rank correlation coefficients reflected weak positive associations, indicating that as *M. hapla* P_i increased, more disease was observed, but were not statistically significant ($\rho \leq 0.239$, $P > 0.05$). Low (≤ 1) levels of damage were also observed in tubers in the non-inoculated plots most likely due to low naturally occurring populations. There was no significant difference between cultivars in RKN damage severity (data not shown).

Table 6.4. Effect of selected initial populations of *Meloidogyne hapla* and *Pratylenchus penetrans* on final populations (P_f) in the potato cvs. Eva and Lamoka in small plot replicated trials conducted in 2017 and 2018 at Freeville, New York State. Means (n = 6) followed by the same letter within each nematode species × year grouping are not significantly different at the 0.05 level for interaction effect of cultivar and initial population density (P_i).

	Year	**2017**		**2018**	
	Cultivar	**Eva**	**Lamoka**	**Eva**	**Lamoka**
Species	**P_i, per pot**				
Meloidogyne hapla	Control (Non-inoculated)	2.8 b	11.1 b	10.0	0.0
	Medium (250)	110.0 b	35.0 b	38.3	16.7
	High (500)	312.5 a	48.6 b	35.0	18.7
	F value =	10.92		0.188	
	P =	0.0003		0.830	
Pratylenchus penetrans	Control (Non-inoculated)	20.1 bc	6.9 c	11.7	8.3
	Medium (500)	27.8 b	25.0 bc	8.3	13.3
	High (1,000)	61.7 a	13.9 bc	10.4	6.7
	F value =	8.68		0.706	
	P =	0.0007		0.504	

***Pratylenchus penetrans*.** Neither main effects of cultivar nor P_i, nor their interaction had a significant effects on yield within the *P. penetrans* block in either year (Table 6.1). Minor but non-significant decreases in average tuber yield were observed within plots inoculated with medium (500 nematodes per plant) and high (1,000 nematodes per plant) P_i densities when compared to control plots, with the exception of Eva plots inoculated with *P. penetrans* in 2017 (Table 6.1). Tuber set was not significantly influenced by either the main or interactive effects of cultivar or P_i in either year of the study (Table 6.2). Tuber diameter was significantly influenced by cultivar in 2017, and Lamoka tubers were 2.9% larger than Eva. Tuber diameter was also significantly influenced by the main effect of P_i in 2017, with the medium P_i (500 per plant) having a 4.7% increase and the high P_i (1,000 per plant) having a 1.3% increase over the non-inoculated control (μ = 78.4 mm). There was no significant interaction between cultivar and P_i on tuber diameter in either year of the study (Table 6.3).

Final population levels of *P. penetrans* were significantly influenced by cultivar and P_i in 2017, with 136% fewer *P. penetrans* recovered from Lamoka than Eva plots (μ = 31.2 nematodes). Medium P_i and high P_i plots produced an average of 95% and 164% more *P. penetrans*, respectively, than the non-inoculated plots (μ = 13.5). Final population levels were significantly influenced by cultivar and P_i in 2017 but not in 2018 (Table 6.4). *P. penetrans* were recovered from soil samples in all inoculated plots in both years of the study.

Low damage scores ($\leq$ 1.83) were observed in the inoculated plots for both cultivars, equating to approximately 4 to 5% of surface area. The Spearman's rank correlation coefficients indicated no significant associations between tuber damage and initial *P. penetrans* populations across both cultivars ($\rho \leq 0.437$, $P > 0.05$). There was minor damage in tubers harvested from the non-inoculated control plots ($\leq$ 1.41), likely

reflective of the presence of low endemic populations. There was no significant difference between cultivars in damage on tubers (data not shown).

Discussion

This study evaluated the response of the two locally popular cvs. Eva and Lamoka to *M. hapla* and *P. penetrans* at three initial population densities typical of those found in New York State intensive cropping rotations. The absence of a significant reduction in tuber weight in both cultivars for the initial populations of either nematode species tested suggested some cultivar tolerance. There was a general trend of minor decreases in tuber set (Table 6.2) and average tuber diameter (Table 6.3) in both cultivars inoculated with either species, suggesting that the minor reductions in total yield may have been due to a combined reduced size and number of tubers. Yet the differences in tuber diameter were of only a few millimeters, and likely does not represent practical significance in commercial potato production.

Other studies have reported that infestation of roots by either *M. hapla* or *P. penetrans* may actually increase root proliferation and root mass growth in some crops when population densities in the soil fall beneath critical thresholds (Bernard and Laughlin 1976; Olthof and Potter 1972; 1977). Jacobsen et al. (1979) observed an increase in foliar biomass of potato infected with *M. hapla* in a greenhouse study. Although no corresponding increase in tuber weight was also recorded, they speculated that the larger plant could potentially support an increase in yield (Jacobsen et al. 1979).

The final nematode population densities measured were low (< 312.5 *M. hapla* J2s and 61.5 *P. penetrans* per 200 ml of soil; Table 6.4) indicating these cultivars may be fair to poor hosts. Although P_f values were low and yield measurements did not indicate a significant yield loss, a build-up of populations over time could occur under a tolerant host cultivar, potentially detrimental to subsequent susceptible potato cultivars or

rotational crops. For example, other vegetable crops commonly grown in New York State such as lettuce, onion, and strawberry are highly sensitive to *M. hapla*, with estimated economic thresholds of 1 to 2 eggs per cm^3 for lettuce (Viaene and Abawi 1996), 200 J2s per 100 cm^3 for onion (Olthof and Barker 1972), and 5 J2s per 100 cm^3 for strawberry (Barker and Nusbaum 1971). Similarly, many rotational crops such as soybean, onion, and corn are also highly sensitive to *P. penetrans*, with estimated economic thresholds of approximately 200 nematodes per 100 cm^3 for soybean (Barker and Olthof 1976), 100 nematodes per 100 cm^3 for onion (Ferris 1962) and 100 nematodes per 100 cm^3 for corn (Olthof and Potter 1973).

No significant crop damage was observed on tubers of cvs. Eva or Lamoka inoculated with either *M. hapla* or *P. penetrans* in this study, also suggesting these cultivars exhibit tolerance to tuber infestation. Previously, thresholds of 100 *M. hapla* J2s and 200 to 600 *P. penetrans* per 100 g soil were defined as the economic thresholds in potato based on field studies (Olthof and Potter 1972; 1973; Barker and Olthof 1976). These previous studies were conducted with older cultivars, which are likely to have differing levels of tolerance than more recently released cultivars. Currently, growers practice prophylactic nematicide application based on these recommendations. These results do not support these recommendations for the cvs. Eva and Lamoka, and current management practices may be leading to a high frequency of false positive decisions. The results also suggest the economic threshold levels for *M. hapla* and *P. penetrans* in Eva and Lamoka are higher than the P_i densities investigated here or those cited in previous studies (Olthof and Potter 1972; 1973; Barker and Olthof 1976; Holgado et al. 2009).

Although this study did not observed many significant effects of P_i on yield loss, crop damage, or final populations, or identified an economic threshold for *M. hapla* or *P. penetrans* in potato production, the results are scientifically important and open new avenues of inquiry into the tolerance or susceptibility of these potato cultivars and others

to plant parasitic nematodes. Furthermore, we believe broader purview should be maintained in light of the results. Melakeberhan et al. (2012) states that in addition to knowledge of initial population densities and cultivar susceptibility, defining economic thresholds for *M. hapla* and other plant parasitic nematodes in potato requires knowledge of the soil conditions, environmental conditions, and degree days affecting the numbers of generations expected within the cropping season. For example, sandier soils could result in increased nematode migration to host roots through larger and homogeneous pore sizes. Moreover, local degree day accumulations may significantly affect the number of generations per season. These factors could result in more substantial crop loss and damage than observed in this study. Yield loss and crop damage may also be influenced by the cultural and chemical management practices being undertaken (Melakeberhan et al. 2012). To the best of our knowledge, this is the first depiction of the responses of potato cvs. Eva and Lamoka to *M. hapla* and *P. penetrans* under field conditions and provides sentinel knowledge to improving the production and profitability of these cultivars in New York State.

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CHAPTER 7

CONCLUSIONS

This thesis sought to quantify the risk of crop loss and damage due to the Northern root-knot nematode (RKN) (*Meloidogyne hapla*) in potato in New York State, by relating initial population densities measured at planting with crop loss and damage at harvest. Additional components also investigated the risk of crop loss due to the root-lesion nematode, *Pratylenchus penetrans*. This work also developed key methodologies and investigated the economic threshold for *M. hapla* in New York State potato production to develop a framework for a pre-plant, DNA-based soil test for quantification of plant-parasitic nematode populations and prediction of risk, with the overarching goal of supporting informed decision making in nematode disease management.

To achieve this goal, a method for isolating nematode DNA directly from 100 g of soil was developed, using super paramagnetic iron oxide nanoparticles (SPION) capture and polyvinylpolypyrrolidone purification. This method was successful in isolating high quality genomic DNA from soil samples. Maximization of DNA captured and minimization of co-capture of contaminants was optimized with the addition of 10 mg SPION. The SPION-based method produced significantly lower quantities of DNA when compared to a standard phenol-based method and a commercial DNA isolation kit. The method was advantageous in not using hazardous materials, economical, and able to process larger volumes of soil, reducing uncertainty in nematode population estimates due to heterogeneity between samples. The lower limit of detection was approximately 100 *M. hapla* second-stage juveniles (J2s) per 100 g of soil (when using a standard PCR reaction that amplified the internal transcribed spacer region of *M.*

hapla), previously regarded as the economic threshold for *M. hapla* in potato production. Work to improve the SPION-based method for enhanced efficiency and extraction of multiple pathogen types for multiplexing purposes may be beneficial. Additionally, research on adaptation of the method for high organic matter soils ('muck' soils) would allow the method to be used for detection of plant-parasitic nematodes in a broad range of vegetable cropping systems relevant to New York State producers.

A species-specific, real-time quantitative PCR (qPCR) assay for the detection and quantification of *M. hapla* using the RKN effector gene *16D10* as a target was developed. Sequences of the *16D10* gene available on NCBI GenBank were aligned and regions of polymorphism explored to generate sets of species-specific qPCR primers, with one set denoted Mha17f and Mha17r identified as having a high degree of species-specificity and optimal reaction parameters. The use of Mha17f and Mha17r in a qPCR assay produced a single amplicon from the coding region of the *16D10* gene. The developed primers and amplification protocol produced positive signals in all five isolates of *M. hapla* tested, and no cross reactivity with 15 additional non-target plant-parasitic nematode species tested. The lower limit of detection for the primers was approximately 5 *M. hapla* J2s per 0.5 g soil using endpoint PCR, and 0.079 pg/µl of *M. hapla* DNA. A high degree of variability in resultant quantification cycles was observed among replicate reactions. This may be explained by the low copy number of *16D10* in *Meloidogyne* spp., which may have led to stochastic partitioning of template sequences into reaction wells. Although only a few complete sequences of the effector gene *16D10* are currently available, an expanded analysis of additional *M. hapla* isolates and other RKN species is needed to understand potential variability within the marker. The qPCR assay served as a proof of concept for using effector genes as species-specific targets for molecular quantification assays. Further long term research may also be devoted to

identifying effector genes that may be used as species-specific markers in other plant-parasitic nematode species affecting potato, including *P. penetrans*.

In 2016 and 2017, the spatial patterns of *M. hapla* and *Pratylenchus* spp. were quantified with standard manual extraction methods and mapped within three commercial potato fields on two occasions. This was accomplished through geostatistical methods including semivariogram analysis, ordinary kriging, and Spatial Analysis by Distance IndicEs (SADIE). Semivariogram analysis indicated initial population densities of *M. hapla* and *Pratylenchus* spp. were spatially dependent over an average range of 110 m and 147 m, respectively. This indicated these distances as the optimal length for placement of sampling points to determine spatial structure. Populations of *Pratylenchus* spp. were significantly aggregated in nearly all fields using the SADIE method (10 of 12 samplings, I_a = 1.367 to 2.113), while *M. hapla* populations were aggregated in few fields (3 of 12 samplings, I_a = 1.318 to 1.738). The Association Function of SADIE was used to conduct a spatiotemporal analysis between initial and final populations of *M. hapla* and *Pratylenchus* spp. within fields, and indicated a significant positive association. *M. hapla* and *Pratylenchus* spp. populations significantly co-associated in only 6 of 12 samplings. This finding suggested that site-specific application of nematicides for control of both taxa would not be economically feasible in many cases. Additional mapping and geostatistical analysis of population densities of *M. hapla* and *Pratylenchus* spp. across a broad area of New York State and the Northeastern United States, including different soil types, crop rotation regimens, and production practices would provide an enhanced knowledge of the epidemiological factors influencing nematode populations in potato production and assist in evaluation of risk.

To quantify the association between initial *M. hapla* populations and potato yield, three commercial potato fields located in western and central New York State were

intensively sampled in in 2016 and 2018. Populations of *M. hapla* were quantified through manual extraction and morphology-based identification and quantification. Additionally, a nested qPCR assay targeting the internal transcribed spacer region of *M. hapla* was developed and used for estimating *M. hapla* populations directly from soil following DNA extraction via the SPION-based method. Initial *M. hapla* populations estimated through the nested qPCR assay were generally higher than initial populations quantified through manual extraction, yet the methods were significantly positively correlated in both years ($r = 0.473$ and 0.333; $P < 0.0001$), indicating the nested qPCR was robust at quantifying relative *M. hapla* population levels from soil. There was no significant association between the initial *M. hapla* populations determined through either method and total yield, tuber number, average tuber diameter, or tuber galling severity. These results suggested the economic threshold for *M. hapla* in the selected potato cultivars (cvs. Envol, Eva, and Lamoka) may be higher than previously regarded at approximately 100 J2s per 100 g of soil. Alternatively these cultivars may have some tolerance to infection without significant reductions in yield or increased crop damage. Future short term research assessing initial *M. hapla* population densities greater than those encountered in this study for their impact on potato yield and crop damage would be useful for defining an economic threshold for *M. hapla* in potato production. Long term research assessing the influence of abiotic factors such as soil temperature or moisture, or biotic factors including different host crops on the aggressiveness of *M. hapla* is needed. The response of commonly grown potato cultivars to additional plant-parasitic nematode species (such as *P. penetrans*) would help to improve site-specific risk assessment for broadacre cropping systems which frequently include several plant-parasitic nematode species.

The response of two potato cultivars commonly grown in New York State to *M. hapla* and *P. penetrans* was tested in a replicated field trial to provide additional data for

refining risk estimates of crop loss and damage based on the cultivars' relative susceptibility or tolerance to infection. Seed pieces of cvs. Eva and Lamoka were inoculated with variable initial population densities of each species, including 250 or 500 *M. hapla* J2s per plant, and 500 or 1,000 *P. penetrans* nematodes per plant. Non-inoculated controls of both cultivars were also included. Yield, tuber set, tuber diameter, and galling severity were assessed at harvest. Final population density under each inoculation regimen was also estimated through manual extraction of nematodes from soil samples at harvest. Initial population density of neither species had a significant effect on total yield. Initial population density of *M. hapla* had a significant effect on tuber set and tuber diameter in 2017, but not in 2018. Disease severity was low (< 5% tuber surface area covered) under all initial population densities in both trials. Initial populations of *M. hapla* and *P. penetrans* had a significant effect on final populations in the first trial, but not the second, with the highest level resulting in significantly greater population densities at harvest than either the medium or non-inoculated controls. The tolerance of these cultivars to these nematode species may facilitate selection of these cultivars in fields where *M. hapla* and *P. penetrans* populations are high. Studies in the short term are needed for assessing additional potato cultivars for their response to *M. hapla* and *P. penetrans*, to provide growers, crop consultants, and researchers with additional information for cultivar selection in fields with high populations.

This research demonstrated that isolating and quantifying nematode DNA directly from soil for population enumeration was equally robust in predicting relative population densities compared to manual standard extraction techniques in the *M. hapla*/potato pathosystem. DNA-based quantification therefore has utility in both nematode research and diagnostic applications. However, the lack of a significant association between initial *M. hapla* populations (quantified either through manual

extraction or DNA-based qPCR quantification) and a reduction in yield components suggests that the previously cited economic threshold of 100 *M. hapla* J2s per 100 g of soil is not accurate for several newly developed potato cultivars preferred by producers in New York State. This conclusion has far-reaching implications regarding the management of *M. hapla*, particularly concerning use of prophylactic nematicides prior to planting and use of tolerant potato cultivars. Results from sampling of commercial fields and from controlled, replicated trials suggest the cvs. Eva, Lamoka, and Envol are susceptible, but tolerant to infection by *M. hapla* and *P. penetrans*. This suggests that application of a pre-plant nematicide for the control of *M. hapla* is likely not needed in the majority of cases because no significant yield loss or crop damage could be associated with elevated initial populations. This potentially negates the need for broadly applied pre-plant nematicides for control of *M. hapla* or *P. penetrans* infection, reducing variable costs of production. In contrast to broadacre, uniform applications of nematicides, site-specific application may be used to treat locations with high initial population densites, when confirmed through manual extraction or DNA-based quantification. Spatial and spatiotemporal analysis of *M. hapla* and *P. penetrans* populaitons provided key information for designing sampling strategies for estimating population spatial structure. Spatial analysis indicated significant aggregation of individual taxa in several cases, but few instances of significant co-occurrence of *M. hapla* and *Pratylenchus* spp., suggesting site-specific nematicide application may not be worthwhile for control of populations simultaneously.

The increases in yield anecdotally reported by New York State producers following a pre-plant nematicide application may be due to suppression of other soilborne pathogens and/or insect pests the early stages of plant growth. Ultimately, further controlled, replicated trials and field surveys evaluating higher initial nematode population densities (e.g., ≥ 10,000 *M. hapla* J2s per plant) could assist in defining an economic

threshold for *M. hapla* in potato production in New York State. Additionally, multi-year studies assessing the effects of carry-over populations from tolerant potato cultivars to more sensitive rotational crops would assist in evaluating long-term economic decisions regarding nematicide application and disease risk.

APPENDIX A

ESTABLISHMENT AND MAINTENANCE OF A HYDROPONICS SYSTEM FOR REARING OF NORTHERN ROOT-KNOT NEMATODE (*MELOIDOGYNE HAPLA*) SECOND-STAGE JUVENILES*

Summary

A hydroponics system was developed and used to generate root-knot nematode (*Meloidogyne hapla*) inoculum for greenhouse, field, and lab experiments. This system was modeled on that used by the program of Dr. Lance Cadle-Davidson at the United State Department of Agriculture, Agricultural Research Service, Geneva, NY. Tomato plants infected with *M. hapla* were placed into hydroponics units. Eggs released from galls on the roots then hatched as infectious second-stage juveniles (J2s) and survived in the reservoir water. The reservoir water was then sieved, the nematodes collected and counted, and inoculum applied where needed.

The alternative method of generating inoculum was to use modified Whitehead trays (wittily referred to as the "pie pan method" for its use of tin pastry baking dishes) to extract nematodes in soil from colonies maintained on tomato in the greenhouse. The "pie pan method" requires considerable effort to produce the same amount of inoculum, takes several days to complete, and the inoculum solution may contain higher numbers of free-living nematodes and other microbes that are not the target pathogen of interest.

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* Gorny, A. M., Cousins, P., Johnston, D., and Pethybridge, S. J. 2019. Establishment and maintenance of a hydroponics system for rearing of the Northern root-knot nematode (*Meloidogyne hapla*) second-stage juveniles. Plant Health Instruc. Submitted for review 3 May 2019.

Introduction

Acquiring a good understanding of the biology of root-knot nematodes (RKN) was imperative for establishing and maintaining the hydroponic system to facilitate the generation of sufficient *M. hapla* populations for inoculations, and for troubleshooting issues with the system.

RKN species are well suited to rearing in a hydroponics system because adult root-knot females maintain a fixed feeding site, the eggs are released outside the roots, and the structure of the roots are maintained during infestation, allowing the roots to maintain their integrity while in the hydroponics system, thus supporting the nematodes' obligate biotrophic lifecycle. RKN molt to the first-stage juvenile within the egg and hatch as a second-stage juveniles (J2s), which is the motile, infectious stage of the lifecycle (Jones et al. 2013). The J2s will locate host roots, penetrate the root, and establish a feeding site (Bridge and Starr 2007; Jones et al. 2013). Males are usually only present in low numbers and thought to develop more frequently under unfavorable environmental conditions (e.g., low oxygen, poor host, elevated temperature, or crowding of J2s) (Eisenback and Triantaphyllou 1991). Hatching is dependent upon moisture and temperature and not initiated by specific root exudates (Jones et al. 2013). The complete lifecycle (egg to egg) for many RKN typically takes 4 to 5 weeks (Bridge and Starr 2007).

Hydroponics System Establishment

Each hydroponics unit (Fig. A.1) consisted of an 18.9 L food-grade plastic bucket with a fitted lid. Two holes were made in the lid, one in the center approximately 2.5 cm in diameter through which the tomato plant stem was placed, and a second 3 cm from the perimeter of the lid, approximately 1 cm in diameter, through which air tubing was threaded. The bucket and lid were painted with black spray-paint to reduce the

amount of light entering the reservoir water to restrict algal growth. An aquarium air pump (18.9 to 56.8 L dual outlet Aqua Culture aquarium pump, Walmart Inc., Bentonville, AR) was attached to a 10.2 cm long bubbling stone with standard diameter aquarium air tubing. The bubbling stone was placed at the base of the bucket and the aquarium pump operated continuously (24 h). The hydroponics unit was placed on a light rack with a 14 h photoperiod and maintained at room temperature (Fig. A.2). The unit was filled with approximately 13 L of distilled water, and amended with 30 ml of liquid fertilizer (Grow Big Hydroponic, Fox Farm Soil & Fertilizer Company, Arcata, CA). A variable amount of 0.5 M calcium hydroxide was added to maintain the pH of the reservoir water between 6.0 and 6.5.

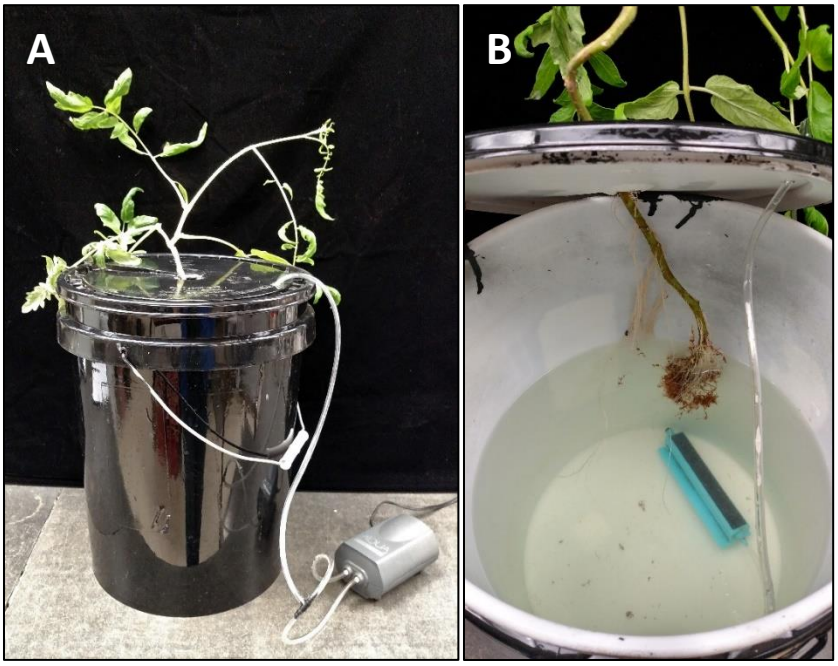

Fig. A.1. Hydroponics unit assembly (**A**) and interior view with root mass and bubbler stone (**B**) for rearing of *Meloidogyne hapla* second-stage juveniles.

Fig. A.2. Lighting rack with four hydroponics units and additional infected tomato plants for rearing *Meloidogyne hapla* second-stage juveniles.

Tomato plants (cv. Rutgers) were seeded on sterilized fine grain beach sand (autoclaved 20 min sterilization time, 5 min dry time). Sand was left to cool on the benchtop, then portioned into 10, 10.2 cm diameter plastic pots and moistened with distilled water. Three seeds were placed in each plot at a depth of approximately 1.5 cm deep and lightly covered. Plants were thinned to one plant per pot when seedlings attained the first set of true leaves. Plants were watered with Miracle-Gro All Purpose Plant Food (The Scotts Company LLC., Marysville, OH) at the manufacturer's recommended rate for the first four weeks after emergence, as the sand did not provide sufficient nutrients for optimal plant growth.

Inoculation of the plants with *M. hapla* J2s began approximately 35 days after planting (DAP), or when the plants were approximately 12 to 15 cm tall. *M. hapla* J2s were

extracted from colonies maintained on tomatoes (cv. Rutgers) in the greenhouse through a modified Whitehead tray method (Whitehead and Hemming 1965). Tomatoes were inoculated by watering the nematode suspension solution into the root zone. Inoculations were repeated every three days for at least 30 days.

After the inoculation period, plants were carefully removed from their pots and the sand washed away with a gentle stream of cool tap water and placed into the hydroponics buckets (one per bucket). Care was taken to support the root mass so that it did not tear away from the plant. The water level was adjusted as needed to ensure the root mass was just covered. Plants were affixed to the light rack with wire ties to support the foliage. Populations of *M. hapla* J2s in the reservoir water were monitored on a weekly basis by taking a sample from the buckets and confirming presence and evaluating populations under 100× magnification.

Maintenance

Each infected tomato plant remained in the hydroponics buckets for approximately 30 days before replacement by a fresh plant, into the same reservoir water. New tomato plants (cv. Rutgers) were seeded every 40 to 50 days to ensure a continuous cycle of plants for inoculation. The replacement tomato plants were inoculated approximately 30 DAP (or when plants were approximately 12 to 15 cm tall) by watering with reservoir water siphoned from the hydroponics buckets. Inoculations continued for at least 30 days. However, depending on the population density of nematodes, inoculation time was extended to ensure establishment of a sufficiently high population. Following the inoculation period, plants were removed from their pots, sand gently washed away, and placed in the hydroponics buckets, as described above.

Rotating new tomato plants through the buckets without replacing the reservoir water for several cycles promoted the build-up of populations. Approximately once every 4 to

5 months, the system components were disassembled, washed with hot soapy water, and reassembled with fresh distilled water. Bleach was not used in the cleaning process as residues may negatively impact nematode populations. After filling the buckets and placing the new tomato plants, 30 ml of liquid fertilizer and a variable amount of 0.5 M calcium hydroxide (to maintain pH of the reservoir between 6.0 and 6.5) was added.

Preparing Inoculum

Working at a sink area, a collection apparatus was assembled (Fig. A.3). A glass funnel was placed over a collection bottle using a ring stand and support. Using a 25 micron mesh sieve held at a 45° angle over the sink, several cups of reservoir water were passed through the sieve. Then, holding the sieve near vertical, nematodes captured on the sieve were washed to one side with distilled water. The back of the sieve was also briefly washed to help retrieve nematodes that passed halfway through the sieve. Finally, nematodes were transferred from the sieve to the collection bottle by turning the sieve slightly over and rinsing into the funnel. The process was repeated until all the water from the hydroponics reservoir passed through the sieve.

Once the nematodes were collected, the concentration of nematodes per ml was determined by removing and counting three aliquots of 3 ml each in a nematode counting dish under 400× magnification. The total quantity of inoculum solution required for a specific objective was then calculated.

Fig. A.3. Funnel apparatus and materials used for collection of nematodes from the reservoir water of the hydroponics buckets.

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